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A Recombinant Truncated Cry1Ca Protein Is Toxic to Lepidopteran Insects and Forms Large Cuboidal Crystals in Insect Cells

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Abstract. A truncated version of the *cry1Ca* gene from *Bacillus thuringiensis* was introduced into the genome of *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) under the control of two promoters. A recombinant virus (vSyncry1c) was isolated and used to infect insect cells in culture and insect larvae. Structural and ultrastructural analysis of insects infected with vSyncry1C showed the formation of large cuboidal crystals inside the cytoplasm of insect cells in culture and in insect cadavers late in infection. Infected insect cell extracts were analyzed by SDS-PAGE and Western blot and showed the presence of a 65-kDa polypeptide probably corresponding to the protease processed form of the toxin. Bioassays using purified recombinant toxin crystals showed a CL_{50} of 19.49 ng/ml for 2nd instar *A. gemmatalis* larvae and 114.1 ng/ml for *S. frugiperda.*

Bacillus thuringiensis (*Bt*) is an entomopathogenic Gram-positive bacteria that produces crystals composed of insecticidal proteins during sporulation. These toxins are called Cry proteins or δ-endotoxins, which can be highly toxic and specific to susceptible insects and at the same time have no toxic effects on other organisms, including other insects [7]. The mode of action of Cry proteins starts with the ingestion of toxin crystals by a susceptible insect, where in the alkaline environment of the midgut, these crystals are dissolved and the protein processed proteolitically to an active form. The activated toxin interacts with proteins in the membrane of columnar cells, forming pores that will cause the loss of cell contents and membrane potential, which leads to cell swelling and death. The insect quickly stops feeding and eventually dies [7]. Cry protein specificity is dependent on the binding of the active form of the toxin to the midgut membrane of susceptible insets [12]. More than 300 Cry protein genes have been cloned and sequenced from different strains of *Bt* [6] and the amino acid sequence of these proteins is the main criteria for their classification [6].

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The *cry1* gene family codes for proteins with a molecular mass of around 130 to 140 kDa, which forms mainly bipyramidal crystals during sporulation [7]. The *cry1Ca* gene was first described by Honée et al. [13] and its product showed toxic activity to *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), *Spodoptera litura* (Fabricius) (Lepidoptera, Noctuidae), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), and *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) [5, 16, 22] and due to their toxicity to these important insect pests, the *cry1Ca* genes have been used for the construction of transgenic plants [5, 16].

The Lepidopteran insect pests that have the most economical impact on the maize culture in México, South and Central America is *Spodoptera frugiperda* (Lepidoptera: Noctuidae). This insect shows low susceptibility to Cry proteins from *B. thuringiensis* subsp. *Kurstaki*, which is usually used for its control [3]. Another important lepidopteran insect pest in Brazil is *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). This insect is the main defoliator pest in soybeans and is highly susceptible to Cry1 toxins [2].

Baculoviruses are biological control agents used in agriculture and also one of the most popular vectors

(BEVs) for heterologous gene expression in insect cells [14], due to the presence of strong promoters (p10 and polyhedrin promoters) that allow a high level of heterologous protein expression late in infection. BEVs have been used to express full-length and truncated forms of Cry proteins from *Bt* [17, 19, 20, 21] and fusion of a Cry protein with the main protein of baculovirus occlusion bodies (OBs) [4]. In the present study, we have introduced a truncated version of the *cry1Ca* gene into the genome of a baculovirus and analyzed the recombinant protein expression in cultured insect cells and insect larvae. Furthermore, we have shown that the recombinant protein produced cuboidal-shaped crystals and was toxic to second instar *S. frugiperda* and *A. gemmatalis* larvae.

Materials and Methods

Cells and Viruses. *Trichoplusia ni* (BTI-Tn5B1-4) [11] cells were maintained at 27°C in TC-100 medium supplemented with 10% fetal bovine serum (Invitrogen). This cell line served as host for the in vitro propagation of *Autographa californica multiple nucelopolyhedrovirus* (AcMNPV) and the recombinant virus vSyngalVI-, which contains the β-galactosidase gene in the polyhedrin locus [18], and was used for the construction of the recombinant baculovirus containing the *cry1Ca* (this work).

Recombinant Vírus Construction. A truncated version (2415 bp) of the *cry1Ca* gene (3570 bp) from the *B. thuringiensis* strain 1644 (Embrapa Maize & Sorghum, Brazil) was amplified by PCR reactions using specific oligonucleotide primers (F1, 5′-CCTTTTAGGATCCTT *ATG*GAG-3′ and R1, 5′-CCAGATCTGCAGGAACAATCTAGATCA GGATTC-3′). The F1 primer anneals at the 5′ end of the *cry1Ca* gene (the start codon of the gene is shown in italics). The R1 primer anneals at 2444 to 2469 nucleotides after the start codon. The *Bam* HI and *Bgl* II restriction site were introduced into the sequence of the primers (underlined sequence). The amplified fragment (67.6% of the full-length gene) was cloned into the plasmid pGEM-T easy (Promega) following the manufacturer's instructions and sequenced (MEGA BACE 1000, Amersham Bioscience). DNA from the recombinant plasmid (pGEMcry1C) was digested with *Eco* RI and separated by electrophoresis in a 0.8% agarose gel. The 2415-bp fragment was gel-purified using the GFX Kit (Amersham) and cloned into the transfer vector pSynXIVVI+X3 previously digested with *Eco* RI [18]. One microgram of the recombinant plasmid (pSyncry1C) DNA and 0.5 μg of vSyngalVI-DNA, previously linearized with the restriction enzyme *Bsu* 36I, were co-transfected into a monolayer of BTI-TN5B1-4 cells (10^6 cells) in a 60-mm plate, using liposomes (Cellfectin® from Invitrogen). The plate was incubated one week at 27°C, when the supernatant was collected and used to purify the recombinant virus through end-point dilution in 96-well plates [18].

Recombinant Protein Expression. Six-well plates (TPP) were seeded with BTI-TN5B1-4 cells (10^6/well) and infected with wild-type AcMNPV and different recombinant viruses (10 pfu/cell). After 1 h (zero times p.i.), virus inoculum was removed and the plates incubated at 27°C. Ninety-six hours p.i, cells were collected by centrifugation (1000*g*/5 min) and stored at −80°C. Thirty third-instar *S. frugiperda* larvae were infected with the recombinant vSyncry1C by injection of virus (5×10^5 pfu) into the hemocoel using a microserynge (Hamilton). At 120 h p.i, dead larvae were collected and kept at −80°C. Infected insect cells and dead insect extracts were suspended in a small volume of PBS (136 mM NaCl, 1,4 mM KH_2PO_4, 2,6 mM KCl, 8 mM $Na2HPO_4.2H_2O$, pH 7.4) and proteins were fractionated in a 12% SDS-PAGE [15] using a Mini-Protean apparatus and following the manufacturer's instructions (Bio-Rad). After electrophoresis, proteins were blotted onto PVDF membranes (Millipore) using the Trans-Blot® SD device, following the manufacturer's instructions (Bio-Rad). A polyclonal antibody raised against the Cry1Ca protein and the Western Breeze™ Immunodetection Kit (Invitrogen) was used to detect the recombinant protein. The polyclonal antibody was produced from gel-purified recombinant Cry1Ca protein.

Structural and Ultra-Structural Analysis of Putative Cry1Ca Crystals Produced in Insect Cells and Larvae. Insect cells were infected with AcMNPV and vSyncry1C as described above and at 96 h p.i., analyzed in a light microscope (Axiovert 100, Zeiss), and photographed. One hundred third-instar *S. frugiperda* larvae were infected with the recombinant vSyncry1C by injection of virus as described above, and at 120 h p.i., OBs and protein crystals were purified following the protocol described by O`Reilly et al. [18]. The purified OBs and crystals were processed for Scanning electron microscopy and analyzed under a Jeol JSM 840 scanning electron microscope at 10-Kv.

Bioassays. Different doses of purified polyhedra and recombinant Cry1Ca protein from insect cadavers (30 μL containing $30{,}000 \times 10^{-3}$, $3{,}000 \times 10^{-3}$, 300×10^{-3}, 30×10^{-3}, 3×10^{-3} , 0.3×10^{-3}, 0.03×10^{-3}, 0.003×10^{-3} , 0.0003×10^{-3} and 0.00003×10^{-3} ng/ml, respectively) were spread over the insect diet and 24 second instar *S. frugiperda* and *A. gemmatalis* were incubated with each dose. The quantification of the recombinant protein was carried out using the Bradford reagent following the manufacturer's instructions (Bio-Rad). Insect mortality was scored at 24 and 48 h post-inoculation. CL_{50} was determined by Probit analysis [9].

Results

Construction and Isolation of the Recombinant Virus vSyncry1C. A truncated *cry1Ca* gene (2415 bp) was cloned into the plasmid pGEMT-easy (not shown) and sequenced. The sequence showed a truncated protein (804 amino acid) identical to the first 824 amino acids of the Cry1Ca protein (1189 amino acids) described by Honée et al. [13] with the exception of a 20–amino acid deletion between amino acids 589 and 608 (not shown). The *cry1Ca* gene was then cloned into the transfer vector pSynXIVVI+X3 (Fig. 1A). The recombinant plasmid pSyncry1C (Fig. 1B) was co-transfected into insect cells with linearized vSynGalVI- DNA (Fig. 1C) and the recombinant vSyncry1C was isolated (Fig. 1D).

Recombinant Protein Expression Analysis. BTI-TN5B1-4 cells were infected with different recombinant viruses and at 96 h p.i. cell extracts were analyzed by SDS-PAGE and Western blot (Fig. 2A and 2B). A protein of around 65 kDa was detected in vSyncry1C-infected cell extracts and vSyncry1C-infected *S. frugiperda* larval extracts using an antibody raised against the Cry1Ca protein (Fig. 2B).

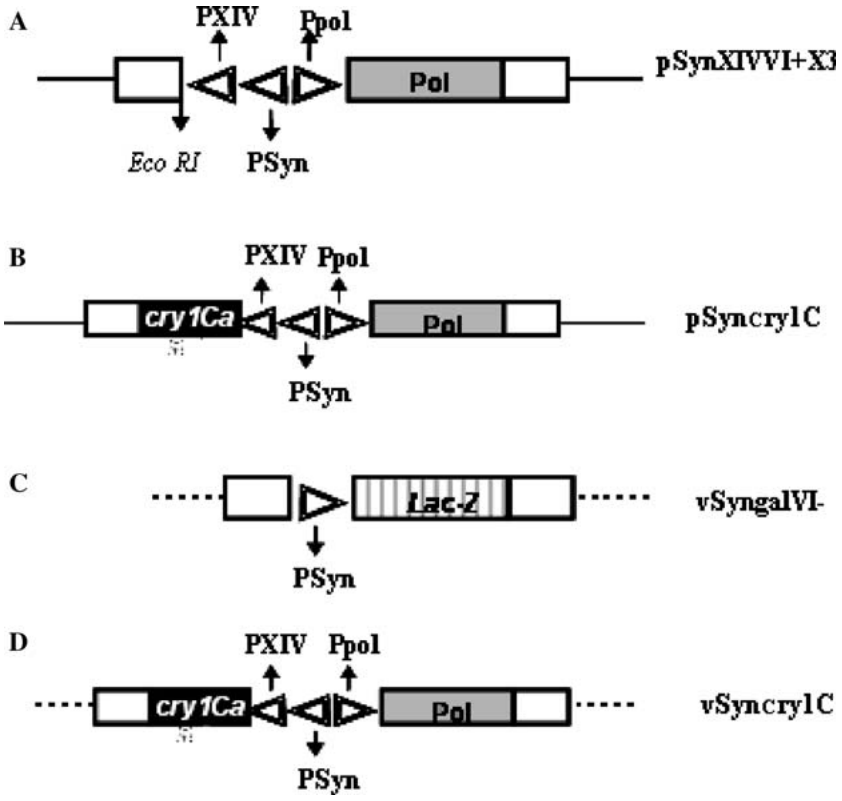

Fig. 1. Diagram showing the polyhedrin locus in the different plasmids and virus used in this work. (**A**) Plasmid pSynXIVVI+X3, (**B**) plasmid pSyncry1C, (**C**) Virus vSyngalVI- and (**D**) Virus vSyncry1C. The *cry1Ca* gene was cloned into the Eco RI site in the plasmid pSynXIVVI+X3 which was used in a co-transfection with DNA from the vSyngalVI- in insect cells for the construction of the recombinant vSyncry1C. The position of the *pol* (polyhedrin), *lac-Z* (β-galactosidase) and *cry1Ca* genes and their promoters (PXIV, Ppol and PSyn) are indicated.

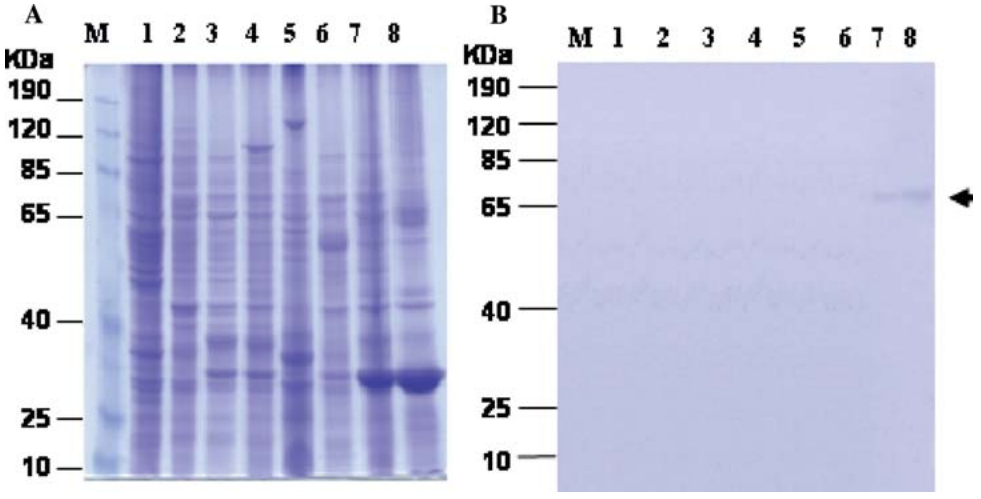

Fig. 2. Recombinant protein expression analysis. (**A**) 12% SDS-PAGE containing mock infected cells (lane 1), and cell extracts from BTI-TN5B1-4 infected with vSyngalVI- (lane 2), AcMNPV (lane 3), vSynprotD (lane 4), vAcBtm (lane 5), vSynYFE (lane 6), and vSyncry1C (lane 7). Lane 8 shows extract from *S. frugiperda* larvae infected with the vSyncry1C. (**B**) Immunoblot of a similar gel shown in A using an antibody raised against the recombinant Cry1C protein. The arrow shows the labeling of a 65 kDa protein in BTI-TN5B1-4 cells and *S. frugiperda* extracts. M is the molecular mass marker (Bench Mark, Invitrogen). The recombinant viruses vSynprotD, vAcBtm and vSynYFE have the protease gene from dengue virus, *cry1Ab* gene from *B. thuringiensis* and envelope protein gene from the yellow fever virus, respectively.

Structural and Ultra-Structural Analysis of Putative Cry1Ca Crystals Produced in Insect Cells and Larvae. *T. ni* cells cells and third-instar *S. frugiperda* larvae infected with the vSyncry1C produced large cuboidal crystals that were visualized by light (Fig. 3A) and scanning electron microscopy (Fig. 3B).

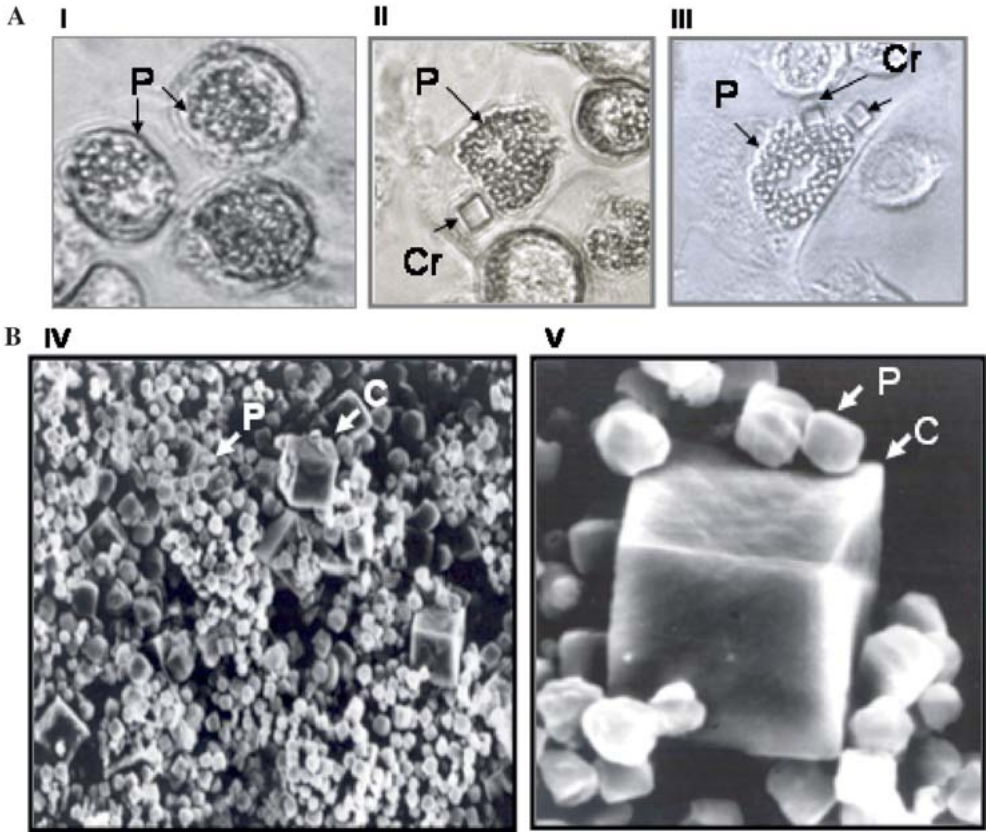

Fig. 3. Structural and ultra-structural analysis of putative Cry1Ca crystals produced in insect cells and larvae. (**A**) Light microscopy of BTI-TN5B1-4 cells infected with AcMNPV (I) at 96 h p.i. and with the recombinant vSyncry1C (II and III). Polyhedra (P) and crystals (Cr) are shown with arrows. (**B**) Ultrastructural analysis of vSynCry1C purified crystals (IV and V). Scanning electron microscopy of purified viral occlusion bodies, also known as polyhedra (P) and crystals (C) from *S. frugiperda* larvae infected with the recombinant vSyncry1C.

Table 1. Bioassay with purified polyhera and crystals from insect cadavers infected with the recombinant virus vSyncrylC.

Insects	CL50 (ng/mL)	Fiducial limits	X^2
S. frugiperda	114.44	32.17/529.62	4.68
A. gemmatalis	19.49	4.91/156.88	2.38

Twenty'second-instar larvae from *A. gemmatalis* and *S. frugiperda* were used for each dose in the bioassay. The results are from three separate experiments.

Bioassays. The CL_{50} for *A. gemmatalis* and *S. frugiperda* were 19.49 ng/ml and 114.44 ng/ml, respectively (Table 1). However, no statistic difference was found since the fiducial limits overlapped. Therefore, both insects are similarly susceptible to the truncated Cry1Ca toxin.

Discussion

A truncated version of a *cry1Ca* gene from *B. thuringiensis* strain 1644 was inserted into the baculovirus AcMNPV genome under the control of two promoters (Psyn and PXIV) arranged in tandem for the construction of the recombinant vSyncry1C. *T. ni* cells and third instar *S. frugiperda* larvae were infected with vSyncry1C and extracts showed a polypeptide, not present in wild type virus and other recombinant viruse-infected cells and insects, of around 65 kDa in SDS-PAGE. This polypeptide probably corresponds to the Cry1Ca protein processed by insect proteases. The Cry1 proteins from *B. thuringiensis* are synthesized as large precursors (protoxins), which are cleaved by insect gut proteases to

form active toxins of around 65 kDa [7]. The expression of *cry* genes in insect cells using BEVs can be an alternative method for the study of single Cry proteins. Some Cry proteins have already been expressed in insect cells using BEVs, such as Cry1Ab, Cry1Ac [20, 21] and Cry11Aa [19], showing that the recombinant proteins were biologically similar to their native counterparts, with toxicity towards different insects. However, the pathogenicity of the recombinant baculovirus was not improved. On the other hand, when the Cry1Ab protein was expressed as a fused protein with the virus main occlusion body protein (polyhedrin), the recombinant virus showed an improvement in its pathogenicity against *P. xylostella* larvae [4].

Although truncated, the recombinant Cry1Ca showed toxicity to second instar *S. frugiperda* CL_{50} 144.44 ng/ml and *A. gemmatalis* CL_{50}-19.49 ng/ml larvae (Table 1). This was expected since a fragment of around 1989 bp coding for a Cry1Ca protein with approximately 663 amino acids was recently expressed in tobacco and protected the plant from *S. litura* [16].

Despite the fact that one third of the Cry1Ca protein (approximately 365 amino acids), expressed in insect cells by the recombinant vSyncry1C, is absent from the C-terminal region and a 20–amino acid deletion between amino acids 589 and 608, of the recombinant protein expressed in this work, the protein was capable of forming large cuboidal crystals in the cytoplasm of infected cells (Fig. 2). The formation of crystals by Cry proteins in insect cells infected with recombinant baculoviruses has been reported for the Cry1Ab, Cry1Ac, and Cry11Aa proteins [17, 19, 20, 21]. These recombinant proteins were expressed in high amounts and some formed larger crystals than when expressed in *Bt*, suggesting that the size of the crystal produced in *Bt* is limited by the size of the bacteria [21]. In a previous work, one of us has shown the formation of crystals from truncated versions of the Cry1Ab protein in insect cells [21]. However, the amounts of crystals were reduced when compared to the expressed full-length protein. Some authors correlate the crystal formation with the presence of cysteine residues in the c-terminal region of the cry proteins, which are responsible for disulfide bond formation and have been suggested to be important in the formation of such crystals [1]. The Cry1Ca protein has 19 cysteine residues and the truncated version expressed in this work has only 11. Therefore, the 8 cysteine residues absent from the truncated Cry1Ca protein are not essential for crystal formation in insect cells. Besides that, recent studies have shown that crystal formation for some Cry proteins is also dependent on the expression of auxiliary proteins [10]. Therefore, it is reasonable to think that proteins from insect cells might help the formation of such large crystals inside the cytoplasm.

Another utility of introducing Cry protein genes into the baculovirus genome is the expression of single Cry proteins in insect cells for functional studies, since these proteins can be studied isolated without the contamination of other Cry proteins that is commonly found in *Bt* strains. Another possible advantage is the formation of large crystals made of Cry proteins [19, 21], which may facilitate structural studies of these proteins. The high level of expression and crystal formation ability of insect cells infected with recombinant baculoviruses carrying Cry protein genes could even facilitate synergistic studies between different toxins. Previous work has shown that Cry proteins can act synergistically, increasing their toxicity towards susceptible insects [22] and these studies will help researchers to choose which genes are more appropriate for being introduced into the genome of economically important crops.

Transgenic *Bt* crops have been commercialized for almost 10 years and several companies have been involved in developing this technology [23]. Transgenic plants expressing insecticidal proteins from *Bt* were grown on over 13 million ha in the United States and 22.4 million ha worldwide in 2004 and this technology is an important tool for the control of lepidopteran pests [24]. Since insects have the potential to develop resistance to Cry proteins [8] and the expression of different Cry proteins in the same plant can reduce or delay the onset of resistance [23], the search for new *cry* genes is an important strategy in order to construct a gene bank of possible candidates for introduction into economically important crops.

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