

Molecular characterization of a Colombian *Bacillus thuringiensis* strain with activity against *Tecia solanivora* (Lepidoptera: Gelechiidae)

Caracterización molecular de una cepa colombiana de *Bacillus thuringiensis* con actividad contra *Tecia solanivora* (Lepidoptera: Gelechiidae)

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Abstract: The Guatemalan potato moth, *Tecia solanivora* (Lepidoptera: Gelechiidae), is one of the most important pests affecting potatoes in northern South America, causing crop losses ranging from 50 to 100%. In this work, we isolated a native strain of *Bacillus thuringiensis* (146-15801) with high activity against this moth, and we characterized its *Cry* genes. This strain was the most active out of eleven strains tested against *T. solanivora* in laboratory bioassays. Specific PCR and genome walk allowed the isolation of *Cry* genes showing 99 and 95% similarity to the sequences reported in *Cry1Ac* and *Cry2* genes, respectively. Immunolocation experiments demonstrated that *Cry1Ac* and *Cry2* bind to the midgut epithelial cells, suggest that these two proteins are involved in the insecticidal activity against *T. solanivora*. Results obtained in this work are relevant because native strains comprise a source of biological components for the development of new bioinsecticides, as well as new *Cry* genes that can be used for biological control.

Key words: Delta-endotoxins. Genome walk. Sequence analysis.

Resumen: La Polilla Guatemalteca de la Papa, *Tecia solanivora* (Lepidoptera: Gelechiidae), es una de las plagas más importantes del cultivo de la papa en el norte de Sur América, causando pérdidas que oscilan entre el 50 y el 100% de la cosecha. En este trabajo aislamos una cepa nativa de *Bacillus thuringiensis* (146-15801) con alta actividad contra esta polilla y caracterizamos sus genes *Cry*. Esta cepa fue la más activa de once cepas evaluadas contra *T. solanivora* en bioensayos en el laboratorio. Técnicas de PCR específica y caminado genómico permitieron el aislamiento de los genes *Cry* mostrando un 99 y 95% de similitud con los genes reportados para *Cry1Ac* y *Cry2*, respectivamente. Experimentos de inmunolocalización demostraron que *Cry1Ac* y *Cry2* se unen a las células epiteliales del intestino medio del insecto, sugiriendo que estas proteínas están involucradas en la actividad insecticida contra *T. solanivora*. Los resultados obtenidos en este trabajo son relevantes debido a que las cepas nativas constituyen una fuente de componentes biológicos para el desarrollo de nuevos bioinsecticidas, así como nuevos genes *Cry* que puedan ser usados para el control biológico.

Palabras clave: Delta-endotoxinas. Caminado genómico. Análisis de secuencia.

Introduction

The Guatemalan Potato Moth (GPM) (*Tecia solanivora* Povolny, 1973) (Lepidoptera: Gelechiidae) was first described in Central America (Povolny 1973) and is currently the most important entomological pest affecting potato (*Solanum tuberosum* L.) in Central America and northern South America (Valderrama *et al.* 2007). Its larvae attack tubers, both in the field and in storage causing losses that range from 50 to 100% (Zeddum *et al.* 2008)

Pest control is usually made by intensive use of chemical insecticides, potentially generating insect resistance and environmentally associated problems (MacLeod 2005; Valderrama *et al.* 2007). *Bacillus thuringiensis* Berliner, 1915 is either a useful alternative or complementary approach to synthetic chemical pesticide applications in commercial agriculture. During sporulation, *B. thuringiensis* cells produce proteina-

ceous crystalline inclusions composed of δ -endotoxins which specifically kill insect larvae. The specificity of these toxins is determined by their binding affinity to the midgut receptors of the apical membrane of brush border epithelial cells. This is followed by insertion into the cell membrane to form pores, causing paralysis and death (Grochulski *et al.* 1995; De Maagd *et al.* 1999). Each type of *Cry* toxin has a unique spectrum of activity and targets only a small range of insect species. Within this small host target range, there are great differences in potency between species that are often closely related (Gilliland *et al.* 2002).

In order to have the best *Cry* proteins for insect control and to prevent or reduce the appearance of insect resistance to current available δ -endotoxins, it is important to identify new *B. thuringiensis* strains containing *Cry* proteins with high toxicity against *T. solanivora*. Although, it has been reported that *Cry1Ac* toxin from *B. thuringiensis* subsp. *kurstaki* is

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active against this insect (Valderrama *et al.* 2007), it is unknown which toxic protein is more specific and which has better activity against *T. solanivora*.

In this study we report the identification of a native *B. thuringiensis* strain isolated in Colombia, with high toxic activity against *T. solanivora*. The strain was characterized by sequence analysis of its *cry* genes and the immunolocalization of the Cry proteins in the intoxicated insect. The availability of this strain and genes provides new alternatives for the control of this insect and their use as control agents for other pests.

Materials and Methods

***Tecia solanivora* larvae.** *Tecia solanivora* (Lepidoptera: Gelechiidae) larvae were obtained from a colony maintained in the laboratory of Plant Biotechnology of the Corporación para Investigaciones Biológicas, (CIB) (Londoño & Montoya 1994).

Strains, culture conditions and protoxin production. Eleven *B. thuringiensis* strains isolated in Colombia, with previously known genotypes, were initially used in this study (Table 1). The *B. thuringiensis* serovar *kurstaki* strain HD-1 was used as positive control. The strains were supplied by the Biotechnology and Biological Control Unit of CIB.

Strains were grown in M1 medium (Orduz *et al.* 1992) at 30°C for 48 h. To solubilize the protoxins this culture was centrifuged at 6000 rpm in a Sorvall-Kendro centrifuge (Ashville, United States), for 10 min, the pellet was resuspended in 50 mM NaOH, 10 mM EDTA solution and incubated at 30°C for 2 h. This solution was centrifuged at 6000 rpm for 15 min at 4°C and the supernatant was collected. Total protein concentration of the solubilized protoxins was determined by Bradford assay (Bradford 1976) using bovine serum albumin as standard.

Bioassays. In order to determine the 50% lethal concentration (LC₅₀), the solubilized protoxins were evaluated using six concentrations starting from 300 ng/ul to 9.4 ng/ul with a 0.6 dilution factor in PBS buffer (Sambrook & Russell 2001). Five replicates of cylindrical pieces of potato (*Solanum tuberosum* var. *capira*) per concentration were immersed once in the solutions for 5 min and allowed to dry at room temperature (25 ± 2°C). These pieces were placed in disposable covered 5 oz plastic cups and each potato piece was infested with five first-instar larvae and left at a constant temperature (23 ± 1°C) and humidity (75 ± 1%) during eight days. After this period, the number of dead larvae was scored, statistically significant differences were identified by Dunnett Test and the LC₅₀s of the toxins were estimated by Probit analysis on at least three independent bioassays (Dunnett 1955; Finney 1971).

PCR amplification of *cry* genes. The most active *B. thuringiensis* native strain (coded 146-15.801) was cultured overnight at 30°C and shaken at 220 rpm in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.0). 1 ml of culture was centrifuged and the pellet was resuspended in 100 µl of distilled water, boiled for 10 min, and spun at 13,000 rpm for 5 min. The supernatant was collected and used as a source of DNA template for PCR amplification using a series of primers based on conserved regions of *cry1Ac* genes (primers cry001 to cry004, Table 1).

For amplification of *cry2* gene, previously described UN2 primers were used (Ben-Dov *et al.* 1997). PCR reactions contained 1 µl of template DNA, 1X reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 µM primer and 1.5 U of *Taq* DNA polymerase (MBI Fermentas, Vilnius, Lithuania). The amplifications were carried out in a iCycler thermocycler (Bio-Rad, CA, USA), with an initial step of 94°C for 3 min, followed by 25 cycles of 92°C for 1 min; 51°C for 30 s and 72°C for 1 min, and an extra cycle with an extension of 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel supplemented with 0.1% ethidium bromide and visualized in a UV transilluminator (Fotodyne Inc., Harhand, WI, USA).

Sequencing of *cry* genes. The PCR products were cloned into pGEM-T Easy Vector System I (Promega, Madison, WI, USA), and sequenced using an ABI 3730 DNA sequencing machine as a service provided by Macrogen (Seoul, Korea). In order to obtain the complete sequence of *cry* genes, cloning of flanking DNA regions was achieved by genome walking (Siebert *et al.* 1995). Briefly, 5 µg of the most active *B. thuringiensis* native strain DNA was digested with four restriction enzymes (*Sma*I, *Eco*RV, *Pvu*II and *Rsa*I) and ligated with genome walker adaptors. The ligated fragments were used for an initial PCR amplification with an outer adaptor-specific primer (AP1) and outer gene-specific primers (12GW1, 12GW2 and Cry2GW1, Cry2GW2) (Table 2). The products of these reactions were used as template for a second PCR amplification with the nested adaptor primer AP2 and nested gene specific primers (12GW3, 12GW4 and Cry2GW3, Cry2GW4) (Table 2). This method produced PCR fragments that extended from the known sequence of *cry* genes into adjacent DNA, both 5' and 3' of the known sequence depending on the location and sequence of gene specific primers. The PCR products were analyzed by 1% agarose gel electrophoresis. The PCR purified products were ligated into pGEM-T Easy Vector System I (Promega) and sequenced. The complete sequences of the encoding region of each gene were confirmed by three independent PCR reactions using high fidelity *Pfu* polymerase (MBI Fermentas).

Sequence analysis. Sequences obtained of *cry1Ac* and *cry2* genes from 146-15.801 strain were manually edited using BIOEDIT Sequence Alignment Editor v7.0.5.2 (Hall 1999), and a multiple alignment was made with the reported sequence of *cry* genes in the NCBI/GenBank. Both nucleotide and amino acid sequence differences were obtained using ClustalW (Hall 1999).

Immunolocalization. An immunolocalization assay was performed with commercial anti Cry1Ac, anti Cry1Ab and anti Cry2A antibodies (Abraxis, Warminste, PA) in order to study the binding of the Cry toxins of the native *Bacillus thuringiensis* strains to the cell membrane of midgut cells of first instar *T. solanivora* larvae. The *B. thuringiensis* serovar *kurstaki* strain HD-1 was used as positive control. Briefly, three days protoxin fed larvae were placed in 100% paraplast blocks and were cut in 5 µm thick slices using a microtome (Leica RM 2135, Suchthilfe Wetzlar, Germany). Slices were incubated in three changes of 100% xylol for 5 min at room temperature and these were dehydrated in an ethanol series of 100%, 85%, 70% and 50% 3 min each at room temperature. Slices were then incubated in 1mg/ml trypsin (Sigma, St Louis, USA)

Table 1. Nucleotide sequence of the primers used in the study.

Primer	Sequence (5' → 3')	Specificity	Purpose
cry001 forward	AATGAATGCATTCTTATAATTG		
cry001 reverse	ATAAATTTCTCTTGTTAATTGGG		
cry002 forward	CCCAATTAACAAGAGAAATTTAT		
cry002 reverse	CTTGATCAATATGATAATCCGT		
cry003 forward	ACGGATTATCATATTGATCAAG	<i>cryIa</i> gene	isolation of coding region
cry003 reverse	CCGCTTGTAATTGATCATATTGAG		
cry004 forward	CTCAATATGATCAATTACAAGCGG		
cry004 reverse	CCTCCTAAGGAGTAATCCACGC		
UN2 forward ^a	GTTATTCTTAATGCAGATGAATGGG		
UN2 reverse ^a	CGGATAAAATAATCTGGGAAATAGT	<i>cry2</i> gene	isolation of coding region
12GW1	CTCCACCTAATACTTCTACTTCAGGG		
12GW2	GGATTGAGATTGGAGAAACGGAAGG		
12GW3	GGTTACTTAAACAATTATAAGG		
12GW4	CGGAAGGAACATTTATCGTG		
Cry2GW1	TGTTGCTGCTGAAATACCCATTCA	gene-specific primers	Genome Walking
Cry2GW2	TGGTGCTTTTTCAGCCCGTGG		
Cry2GW3	CCCCATTCTGCATTAAGAATAAC		
Cry2GW4	CGTGGAATTCAACTATTCCAG		
AP1 ^b	GGATCCTAATACGACTCACTATAGGGC	Genome Walker adapter	Genome Walking
AP2 ^b	AATAGGGCTCGAGCGGC		
Cry2CBT12-1 For	TGTGGGTCGTAATAAGCACTTT		
Cry2CBT12-1 Rev	CGTTTCGGTTAGGGTTCAAA		
Cry2CBT12-2 For	TTTGAACCCTAACCGAAACG		
Cry2CBT12-2 Rev	TGGCCAGTTTTGTGCTGTAA	<i>cry2</i> gene	isolation of coding region
Cry2CBT12-3 For	TTACAGCACAAAACCTGGCCA		
Cry2CBT12-3 Rev	GCGCCAAATGGATCATAGTA		
Cry2CBT12-4 For	TACTATGATCCATTTGGGCGC		
Cry2CBT12-4 Rev	TTAGTTCCGTCATATGTTTAAGA		

^a Ben-Dov *et al.* 1997. ^b Siebert *et al.* 1995.

(in 2 N HCl) followed by incubation with 0.1 mM PMSF (Phenyl Methyl Sulfonyl Fluoride) (Sigma, St Louis, USA) for 5 min at room temperature. A treatment with hydrogen peroxide (H₂O₂) at 0.5% in ethanol, for 30 min followed by another treatment at 6% for 15 min was used to eliminate endogenous peroxidases.

Antibody treatment started with one wash in distilled water for 3 min, followed by three washes with TBS (100 mM Tris-HCl, pH 7.5, 0.9% NaCl and 0.1% Tween 20) 3 min each. Each slice was blocked with 3% BSA (Promega) for 1 h at room temperature followed by incubation of 1 h at 37°C in a humidity chamber with a 1:1000 dilution of the antibody in TBS (100 mM Tris-HCl, pH 7.5, 0.9% NaCl and 0.1% Tween 20) with 1% BSA. Negative controls were incubated only with TBS. After three washes with TBS, peroxidase conjugated anti-rabbit antibody was used at a dilution of 1:2.500 and incubated for 40 min at 37°C followed by another three washes with TBS. Development was done with 20 µl of DAB cromogen (DAKO; Denmark) in 1 ml DAB buffer (Imidazole - HCl buffer pH 7.5) for 3 min in the dark at room temperature. For contrast and cell identification each

preparation was incubated for 2 s in hematoxylin (Sigma, St Louis, USA) and washed in distilled water. The preparations were let to dry at room temperature and observed under light microscopy.

Results

Toxicity of *B. thuringiensis* Cry proteins to *T. solanivora*. Eleven strains were selected for testing based on their *cry* genes (Table 2) in order to find *B. thuringiensis* strains with good activity against *T. solanivora*. Analysis of variance indicated that the average dead larvae was different between the strains ($F = 1.92$; $\alpha = 0.05$; $P < 0.05$). Dunnett test (Dunnett 1955), showed that these differences greater than 1.25 in absolute value relative to the control were significant. *In vivo* toxicity tests indicated that there were differences among the strains tested and two of them, 146-16.203 and 146-15.801, presented the highest activity (Table 2). These two strains were more active than the positive control *B. thuringiensis* subsp. *kurstaki*. Probit analysis of the strains showed that strain 146-15.801, with a previously known *cryIa* and *cry2*

genotype, had the highest toxic activity with an LC₅₀ of 56.82 ng/ul (Table 2). This strain was selected for the isolation and sequencing of its *cry* genes.

Isolation and sequence analysis of *cry1Ac* and *cry2* genes.

Gene specific PCR amplifications and genome walking allowed the isolation and sequencing of a 3.534 nucleotide fragment from strain 146-15.801 encoding for a *cry1Ac* gene; (*Bacillus thuringiensis* toxin nomenclature committee: *cry-*

1Ac24; GenBank Accession EF094884). Comparison of the nucleotide sequence of this gene with reported *cry1Ac* genes: GenBank Accessions AF492767, AY225453, AY122057, U89872, M11068, M73249 and AY730621; revealed, as expected, a high homology among them, but also some nucleotide differences (Fig. 1).

The amino acid sequence deduced from *cry1Ac24* gene showed 99% similarity to the reported proteins. However, most Cry1Ac proteins of the GenBank display an isoleucine

cry1Ac CBT12	1	-----ATGGATAACAATCCGAACATCAATGAATGCAATCTTATAATTGTTAAAGTAACCTGAAAGTAGAAGTATTAGGT	75
cry1Ac Yao, J	1	-----ATGGATAACAATCCGAACATCAATGAATGCAATCTTATAATTGTTAAAGTAACCTGAAAGTAGAAGTATTAGGT	75
cry1Ac Bower, G	1	-----ATGGATAACAATCCGAACATCAATGAATGCAATCTTATAATTGTTAAAGTAACCTGAAAGTAGAAGTATTAGGT	75
cry1Ac, Tzeng, C	101	TTAATAAAAAGAGATGGAGCTAACTATGGATAACAATCCGAACATCAATGAATGCAATCTTATAATTGTTAAAGTAACCTGAAAGTAGAAGTATTAGGT	200
cry1Ac, Gleave, A P	1	-----ATGGATAACAATCCGAACATCAATGAATGCAATCTTATAATTGTTAAAGTAACCTGAAAGTAGAAGTATTAGGT	75
cry1Ac, Adang M	1	-----ATGGATAACAATCCGAACATCAATGAATGCAATCTTATAATTGTTAAAGTAACCTGAAAGTAGAAGTATTAGGT	75
cry1Ac Feitelson, J	1	-----ATGGATAACAATCCGAACATCAATGAATGCAATCTTATAATTGTTAAAGTAACCTGAAAGTAGAAGTATTAGGT	75
cry1Ac, Zhao, Y	1	-----ATGGATAACAATCCGAACATCAATGAATGCAATCTTATAATTGTTAAAGTAACCTGAAAGTAGAAGTATTAGGT	75
cry1Ac CBT12	376	TTAAGAGAAGAGATGCGTATTCAATTC AATGACATGAACAGTGCCTTACAAACCGGTATTCCTCTTTTGCAGTTCAAAATATCAAGTTCCTCTTTTAT	475
cry1Ac Yao, J	376	TTAAGAGAAGAGATGCGTATTCAATTC AATGACATGAACAGTGCCTTACAAACCGGTATTCCTCTTTTGCAGTTCAAAATATCAAGTTCCTCTTTTAT	475
cry1Ac Bower, G	376	TTAAGAGAAGAGATGCGTATTCAATTC AATGACATGAACAGTGCCTTACAAACCGGTATTCCTCTTTTGCAGTTCAAAATATCAAGTTCCTCTTTTAT	475
cry1Ac, Tzeng, C	501	TTAAGAGAAGAGATGCGTATTCAATTC AATGACATGAACAGTGCCTTACAAACCGGTATTCCTCTTTTGCAGTTCAAAATATCAAGTTCCTCTTTTAT	600
cry1Ac, Gleave, A P	376	TTAAGAGAAGAGATGCGTATTCAATTC AATGACATGAACAGTGCCTTACAAACCGGTATTCCTCTTTTGCAGTTCAAAATATCAAGTTCCTCTTTTAT	475
cry1Ac, Adang M	376	TTAAGAGAAGAGATGCGTATTCAATTC AATGACATGAACAGTGCCTTACAAACCGGTATTCCTCTTTTGCAGTTCAAAATATCAAGTTCCTCTTTTAT	475
cry1Ac Feitelson, J	376	TTAAGAGAAGAGATGCGTATTCAATTC AATGACATGAACAGTGCCTTACAAACCGGTATTCCTCTTTTGCAGTTCAAAATATCAAGTTCCTCTTTTAT	475
cry1Ac, Zhao, Y	376	TTAAGAGAAGAGATGCGTATTCAATTC AATGACATGAACAGTGCCTTACAAACCGGTATTCCTCTTTTGCAGTTCAAAATATCAAGTTCCTCTTTTAT	475
cry1Ac CBT12	476	CAGTATATGTTCAAGCTGCAAAATTTACATTTATCACTTTTGGAGAGATGTTTCAGTGTGGACAAGGTGGGGATTTGATGCCGCCGACTATCAATAGTCG	575
cry1Ac Yao, J	476	CAGTATATGTTCAAGCTGCAAAATTTACATTTATCACTTTTGGAGAGATGTTTCAGTGTGGACAAGGTGGGGATTTGATGCCGCCGACTATCAATAGTCG	575
cry1Ac Bower, G	476	CAGTATATGTTCAAGCTGCAAAATTTACATTTATCACTTTTGGAGAGATGTTTCAGTGTGGACAAGGTGGGGATTTGATGCCGCCGACTATCAATAGTCG	575
cry1Ac, Tzeng, C	601	CAGTATATGTTCAAGCTGCAAAATTTACATTTATCACTTTTGGAGAGATGTTTCAGTGTGGACAAGGTGGGGATTTGATGCCGCCGACTATCAATAGTCG	700
cry1Ac, Gleave, A P	476	CAGTATATGTTCAAGCTGCAAAATTTACATTTATCACTTTTGGAGAGATGTTTCAGTGTGGACAAGGTGGGGATTTGATGCCGCCGACTATCAATAGTCG	575
cry1Ac, Adang M	476	CAGTATATGTTCAAGCTGCAAAATTTACATTTATCACTTTTGGAGAGATGTTTCAGTGTGGACAAGGTGGGGATTTGATGCCGCCGACTATCAATAGTCG	575
cry1Ac Feitelson, J	476	CAGTATATGTTCAAGCTGCAAAATTTACATTTATCACTTTTGGAGAGATGTTTCAGTGTGGACAAGGTGGGGATTTGATGCCGCCGACTATCAATAGTCG	575
cry1Ac, Zhao, Y	476	CAGTATATGTTCAAGCTGCAAAATTTACATTTATCACTTTTGGAGAGATGTTTCAGTGTGGACAAGGTGGGGATTTGATGCCGCCGACTATCAATAGTCG	575
cry1Ac CBT12	576	TTATAATGATTTAACTAGGCTTTATGGCAACTATACAGATTTATGCTGTGCTGTTACAATACGGGATTTAGATGTTGGGGACCGGATTTCTAGAGAT	675
cry1Ac Yao, J	576	TTATAATGATTTAACTAGGCTTTATGGCAACTATACAGATTTATGCTGTGCTGTTACAATACGGGATTTAGATGTTGGGGACCGGATTTCTAGAGAT	675
cry1Ac Bower, G	576	TTATAATGATTTAACTAGGCTTTATGGCAACTATACAGATTTATGCTGTGCTGTTACAATACGGGATTTAGATGTTGGGGACCGGATTTCTAGAGAT	675
cry1Ac, Tzeng, C	701	TTATAATGATTTAACTAGGCTTTATGGCAACTATACAGATTTATGCTGTGCTGTTACAATACGGGATTTAGATGTTGGGGACCGGATTTCTAGAGAT	800
cry1Ac, Gleave, A P	576	TTATAATGATTTAACTAGGCTTTATGGCAACTATACAGATTTATGCTGTGCTGTTACAATACGGGATTTAGATGTTGGGGACCGGATTTCTAGAGAT	675
cry1Ac, Adang M	576	TTATAATGATTTAACTAGGCTTTATGGCAACTATACAGATTTATGCTGTGCTGTTACAATACGGGATTTAGATGTTGGGGACCGGATTTCTAGAGAT	675
cry1Ac Feitelson, J	576	TTATAATGATTTAACTAGGCTTTATGGCAACTATACAGATTTATGCTGTGCTGTTACAATACGGGATTTAGATGTTGGGGACCGGATTTCTAGAGAT	675
cry1Ac, Zhao, Y	576	TTATAATGATTTAACTAGGCTTTATGGCAACTATACAGATTTATGCTGTGCTGTTACAATACGGGATTTAGATGTTGGGGACCGGATTTCTAGAGAT	675
cry1Ac CBT12	676	TGGGTAAGGTATAATCAATTTAGAAGAAATTAACACTAACTGTAITAGATATCGTTGCTGTTTGAATATGATAGTTGAAATGATCCAATTCGAA	775
cry1Ac Yao, J	676	TGGGTAAGGTATAATCAATTTAGAAGAAATTAACACTAACTGTAITAGATATCGTTGCTGTTTGAATATGATAGTTGAAATGATCCAATTCGAA	775
cry1Ac Bower, G	676	TGGGTAAGGTATAATCAATTTAGAAGAAATTAACACTAACTGTAITAGATATCGTTGCTGTTTGAATATGATAGTTGAAATGATCCAATTCGAA	775
cry1Ac, Tzeng, C	801	TGGGTAAGGTATAATCAATTTAGAAGAAATTAACACTAACTGTAITAGATATCGTTGCTGTTTGAATATGATAGTTGAAATGATCCAATTCGAA	900
cry1Ac, Gleave, A P	676	TGGGTAAGGTATAATCAATTTAGAAGAAATTAACACTAACTGTAITAGATATCGTTGCTGTTTGAATATGATAGTTGAAATGATCCAATTCGAA	775
cry1Ac, Adang M	676	TGGGTAAGGTATAATCAATTTAGAAGAAATTAACACTAACTGTAITAGATATCGTTGCTGTTTGAATATGATAGTTGAAATGATCCAATTCGAA	775
cry1Ac Feitelson, J	676	TGGGTAAGGTATAATCAATTTAGAAGAAATTAACACTAACTGTAITAGATATCGTTGCTGTTTGAATATGATAGTTGAAATGATCCAATTCGAA	775
cry1Ac, Zhao, Y	676	TGGGTAAGGTATAATCAATTTAGAAGAAATTAACACTAACTGTAITAGATATCGTTGCTGTTTGAATATGATAGTTGAAATGATCCAATTCGAA	775
cry1Ac CBT12	1176	CTCTCAAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCCGCTGGATGAAATACCCACAGAAATAACACCTGCCACCTAGGCAAGGA	1275
cry1Ac Yao, J	1176	CTCTCAAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCCGCTGGATGAAATACCCACAGAAATAACACCTGCCACCTAGGCAAGGA	1275
cry1Ac Bower, G	1176	CTCTCAAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCCGCTGGATGAAATACCCACAGAAATAACACCTGCCACCTAGGCAAGGA	1275
cry1Ac, Tzeng, C	1301	CTCTCAAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCCGCTGGATGAAATACCCACAGAAATAACACCTGCCACCTAGGCAAGGA	1400
cry1Ac, Gleave, A P	1176	CTCTCAAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCCGCTGGATGAAATACCCACAGAAATAACACCTGCCACCTAGGCAAGGA	1275
cry1Ac, Adang M	1176	CTCTCAAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCCGCTGGATGAAATACCCACAGAAATAACACCTGCCACCTAGGCAAGGA	1275
cry1Ac Feitelson, J	1176	CTCTCAAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCCGCTGGATGAAATACCCACAGAAATAACACCTGCCACCTAGGCAAGGA	1275
cry1Ac, Zhao, Y	1176	CTCTCAAAATTTGCCATCCGCTGTATACAGAAAAAGCGGAACGGTAGATTCCGCTGGATGAAATACCCACAGAAATAACACCTGCCACCTAGGCAAGGA	1275
cry1Ac CBT12	1276	TTTAGTCATCGATTAAAGCCATGTTTCAATGTTTCGTTCCAGGCTTTAGT---AGTAGTGAAGTATAATAAGAGCTCCTATGTTCTCTGGATACATCGTA	1372
cry1Ac Yao, J	1276	TTTAGTCATCGATTAAAGCCATGTTTCAATGTTTCGTTCCAGGCTTTAGT---AGTAGTGAAGTATAATAAGAGCTCCTATGTTCTCTGGATACATCGTA	1372
cry1Ac Bower, G	1276	TTTAGTCATCGATTAAAGCCATGTTTCAATGTTTCGTTCCAGGCTTTAGTAAATAGTAGTGAAGTATAATAAGAGCTCCTATGTTCTCTGGATACATCGTA	1372
cry1Ac, Tzeng, C	1401	TTTAGTCATCGATTAAAGCCATGTTTCAATGTTTCGTTCCAGGCTTTAGTAAATAGTAGTGAAGTATAATAAGAGCTCCTATGTTCTCTGGATACATCGTA	1497
cry1Ac, Gleave, A P	1276	TTTAGTCATCGATTAAAGCCATGTTTCAATGTTTCGTTCCAGGCTTTAGTAAATAGTAGTGAAGTATAATAAGAGCTCCTATGTTCTCTGGATACATCGTA	1375
cry1Ac, Adang M	1276	TTTAGTCATCGATTAAAGCCATGTTTCAATGTTTCGTTCCAGGCTTTAGTAAATAGTAGTGAAGTATAATAAGAGCTCCTATGTTCTCTGGATACATCGTA	1375
cry1Ac Feitelson, J	1276	TTTAGTCATCGATTAAAGCCATGTTTCAATGTTTCGTTCCAGGCTTTAGTAAATAGTAGTGAAGTATAATAAGAGCTCCTATGTTCTCTGGATACATCGTA	1375
cry1Ac, Zhao, Y	1276	TTTAGTCATCGATTAAAGCCATGTTTCAATGTTTCGTTCCAGGCTTTAGTAAATAGTAGTGAAGTATAATAAGAGCTCCTATGTTCTCTGGATACATCGTA	1375
cry1Ac CBT12	1373	GTGCTGAATTTAATAATAATAATGTCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGAAACTTTCTTTTAAATGGTCTGTAATTTTCAGGACCAGG	1472
cry1Ac Yao, J	1373	GTGCTGAATTTAATAATAATAATGTCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGAAACTTTCTTTTAAATGGTCTGTAATTTTCAGGACCAGG	1472
cry1Ac Bower, G	1376	GTGCTGAATTTAATAATAATAATGTCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGAAACTTTCTTTTAAATGGTCTGTAATTTTCAGGACCAGG	1475
cry1Ac, Tzeng, C	1498	GTGCTGAATTTAATAATAATAATGTCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGAAACTTTCTTTTAAATGGTCTGTAATTTTCAGGACCAGG	1597
cry1Ac, Gleave, A P	1376	GTGCTGAATTTAATAATAATAATGTCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGAAACTTTCTTTTAAATGGTCTGTAATTTTCAGGACCAGG	1475
cry1Ac, Adang M	1376	GTGCTGAATTTAATAATAATAATGTCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGAAACTTTCTTTTAAATGGTCTGTAATTTTCAGGACCAGG	1475
cry1Ac Feitelson, J	1376	GTGCTGAATTTAATAATAATAATGTCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGAAACTTTCTTTTAAATGGTCTGTAATTTTCAGGACCAGG	1475
cry1Ac, Zhao, Y	1376	GTGCTGAATTTAATAATAATAATGTCATCGGATAGTATTACTCAAATCCCTGCAGTGAAGGAAACTTTCTTTTAAATGGTCTGTAATTTTCAGGACCAGG	1475
cry1Ac CBT12	1473	ATTACTGTTGGGGACTTAGTTAGATTAAATAGTAGTGAAGAAATAATTTTCAAGATAGAGGGTATATTGAAGTCCAAATTCACCTTCCCATCGACATCTACC	1572
cry1Ac Yao, J	1473	ATTACTGTTGGGGACTTAGTTAGATTAAATAGTAGTGAAGAAATAATTTTCAAGATAGAGGGTATATTGAAGTCCAAATTCACCTTCCCATCGACATCTACC	1572
cry1Ac Bower, G	1476	ATTACTGTTGGGGACTTAGTTAGATTAAATAGTAGTGAAGAAATAATTTTCAAGATAGAGGGTATATTGAAGTCCAAATTCACCTTCCCATCGACATCTACC	1575
cry1Ac, Tzeng, C	1598	ATTACTGTTGGGGACTTAGTTAGATTAAATAGTAGTGAAGAAATAATTTTCAAGATAGAGGGTATATTGAAGTCCAAATTCACCTTCCCATCGACATCTACC	1697
cry1Ac, Gleave, A P	1476	ATTACTGTTGGGGACTTAGTTAGATTAAATAGTAGTGAAGAAATAATTTTCAAGATAGAGGGTATATTGAAGTCCAAATTCACCTTCCCATCGACATCTACC	1575
cry1Ac, Adang M	1476	ATTACTGTTGGGGACTTAGTTAGATTAAATAGTAGTGAAGAAATAATTTTCAAGATAGAGGGTATATTGAAGTCCAAATTCACCTTCCCATCGACATCTACC	1575
cry1Ac Feitelson, J	1476	ATTACTGTTGGGGACTTAGTTAGATTAAATAGTAGTGAAGAAATAATTTTCAAGATAGAGGGTATATTGAAGTCCAAATTCACCTTCCCATCGACATCTACC	1575
cry1Ac, Zhao, Y	1476	ATTACTGTTGGGGACTTAGTTAGATTAAATAGTAGTGAAGAAATAATTTTCAAGATAGAGGGTATATTGAAGTCCAAATTCACCTTCCCATCGACATCTACC	1575

Figure 1. Comparison of the nucleotide sequence of *cry1Ac24* gene (*cry1Ac* CBT-12) gene with the reported *cry1Ac* genes: GenBank Accessions AF492767 (Yao, J), AY225453 (Bower, G), AY122057 (Tzeng, C.), U89872 (Gleave, A. P.), M11068 (Adang, M. J.), M73249 (Feitelson, J. S.) and AY730621 (Zhao, Y). Nucleotide differences are indicated by boxes.

Table 2. *B. thuringiensis* strains isolated in Colombia selected for bioassays and LC₅₀s of the solubilized protoxins from these strains against *Tecia solanivora*.

CODE	GENOTYPE	LC50 (ng/ul)	Level of confidence
146-15504	<i>cryIAa, cryIAb, cryIAc, cryIB</i>	94.60	45.43 < LC < 159.80
146-15601	<i>cry4Aa, cry2</i>	84.54	56.00 < LC < 122.42
146-15507	<i>cryIAa</i>	113.77	58.75 < LC < 205.39
146-15516	<i>cryIAa, cryIAb, cryIAc, cryIB, cryID</i>	94.60	45.43 < LC < 159.80
147-0304	<i>cryIAa, cryIAc, cryIB, cryID</i>	214.21	109.41 < LC < 3221.67
146-15538	<i>cryIAa, cryIB</i>	94.60	45.47 < LC < 159.80
146-16203*	<i>cryIAa, cry2</i>	72.27	49.25 < LC < 98.84
147-11007	<i>cryIAa, cryIAc, cryIB</i>	136.54	74.94 < LC < 270.84
147-7801	<i>cryIAa, cryIAb, cryIA, cryIE</i>	113.77	58.75 < LC < 205.39
147-3003	<i>cryIB, cryIC, cryID</i>	214.22	109.41 < LC < 3221.67
146-15801*	<i>cryIAc, cry2</i>	56.82	42.33 < LC < 71.64
HD-1	Kurstaki	77.05	56.27 < LC < 100.48

* Statistical analysis of the bioassays results by Test of Dunnett. These strains showed differences with the negative control, greater than 1.25 in absolute value and it was considered statistically significant.

at position 11, while the 146-15.801 native Cry1Ac protein has an asparagine instead; the reported Cry1Ac proteins show a phenylalanine at position 148, the native Cry1Ac24 protein presents a leucine; the reported Cry1Ac proteins present a proline at position 248, while the 146-15.801 native Cry1Ac24 protein presents a serine; the reported Cry1Ac proteins present an asparagine at position 442, the native Cry1Ac24 protein has no amino acid at this position; and finally at position 507 all proteins exhibit a isoleucine whereas the native Cry1Ac24 protein present a phenylalanine.

In a similar way to the *cryIac24* gene, specific PCR gene amplifications and genome walking allowed the isolation and sequencing of a 1902 nucleotides fragment from strain 146-15.801 coding for a 2 gene (*Bacillus thuringiensis* toxin nomenclature committee: *cry2Aa13*; GenBank Accession EF094885). Comparison with the reported *cry2A* genes: GenBank Accessions AF200816, M31738, D86064, AF433645, AF441855, M23724, X55416, AF164666, AF336115, AY297091 and X57252, revealed that unlike the *cryIac* genes, there is a high variability among the *cry2* genes (Fig. 2). Nucleotide differences observed were dispersed throughout the coding region of *cry2* gene. The amino acid sequence deduced from *cry2Aa13* gene showed 94% similarity to reported proteins.

Sequence comparison showed that most of Cry2 proteins in the GenBank present more than 50 amino acid differences

throughout the protein, not only with the native protein, but also between Cry2 proteins reported.

Inmunolocalization. When *T. solanivora* larvae were fed with 146-15.801 strain or *B. thuringiensis* subsp. *kurstaki* strains used as a positive control, there were marked differences in the binding of the toxins to the microvillar brush border of the epithelial cells throughout the midgut of the larvae (Table 3). Cry1Ac24 was the best binding protein followed by Cry2Aa13. Figure 3 shows the binding of the different toxins to the midgut epithelial cells.

Discussion

In this work we looked for the implementation of an efficient control mechanism for *Tecia solanivora* by identifying a native strain of *B. thuringiensis* with high activity, because few studies have reported which toxin from *B. thuringiensis* have potential activity against this moth (Valderrama *et al.* 2007). We found a Colombian strain with high activity against *Tecia solanivora*. Genes coding for δ -endotoxins were isolated and characterized. Differences in their nucleotide and amino acid sequences with *cryIac* and *cry2A* genes reported were determined. Furthermore we studied the binding of the different *B. thuringiensis* toxins to the midgut of the insect by immunolocalization.

Eleven strains tested in this study showed activity against *T. solanivora*. Nevertheless, strains 146-16.203 (*cryIAa, cry2*) and 146-15801 (*cryIAc, cry2*) showed the highest activity against the insect. Amongst the two strains selected, 146-15.801 strain showed the lowest LC₅₀ and for this reason was chosen as a source to isolate and to study its *cryIac* and *cry2* δ -endotoxin genes. The *cryIac* and *cry2* genes of the most active strains are consistent with previous reports showing that Cry1Ac toxin has good activity against *T. solanivora* (Valderrama *et al.* 2007).

Multiple sequence alignment between the 3.534 nucleotides of 146-15.801 *cryIac24* gene and other reported

Table 3. Detection of Cry proteins during the immunolocalization assay with anti Cry1Ac and Cry2A antibodies.

Larvae fed with strain	Cry1Ac	Cry2A	Cry1Ab	Negative
146-15801	+++a	++b	-	-
<i>Bt kurstaki</i>	+++	++	++	-
PBS	-	-	-	-

Union of the antibodies: +++=high, ++=average, +=low, - = no reaction

cryIac genes showed a 99% homology. Nevertheless, small differences were found located in specific zones previously described to have variability in these genes (Crickmore *et al.* 1998; Schnepf *et al.* 1998). Multiple sequence alignment showed a much higher variability spread in *cry2Aa13* gene when compared to *cryIac24* sequence.

Cry1 proteins have five conserved blocks located in three functional domains: domain I is involved in the pore formation of the epithelial cell membrane; domain II is involved in receptor binding; and domain III is related to the resistance or susceptibility of the toxin to the proteases of the insect, giving stability to the structure and involved in receptor binding (Peferoen 1997; Crickmore *et al.* 1998; Schnepf *et al.* 1998). The amino acid variations found in the Cry1Ac native

protein were located in the domain I (L148; S248), domain II (absence of N442) and domain III (F507). Although it is not possible to say that these variations of Cry1Ac24 native protein affect the functionality of the toxin, these could be responsible for the high activity of the strain against *T. solanivora*. Several studies have shown that the functionality of each domain varies selectively against different insects when point mutations occur (Peferoen 1997; Crickmore *et al.* 1998; Schnepf *et al.* 1998).

Concerning Cry2Aa13 protein, when it was compared to Cry2A proteins published, we found 45 amino acids that were different. This high variability could be related to a higher host range or to the fact that it is involved in dual activity against lepidopterans and dipterans (Peferoen 1997; Crick-

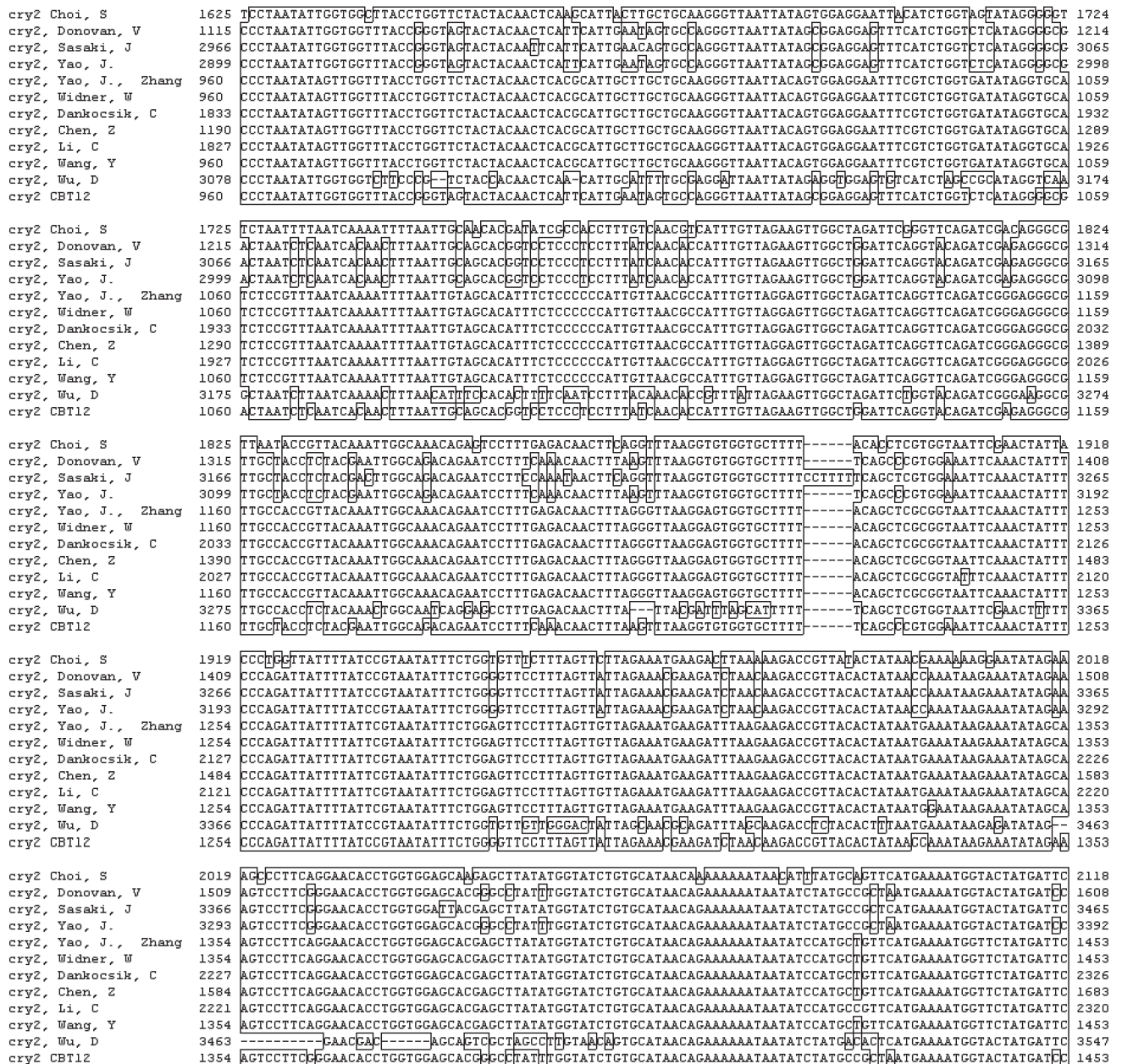


Figure 2. Comparison of the nucleotide sequence of *cry2Aa13* gene (cry2 Pfu CBT-12) with the reported *cry2A* genes: GenBank Accessions AF200816 (Choi), M31738 (Donovan), D86064 (Sasaki), AF433645 (Yao J.), AF441855 (Yao J., Thang), M23724 (Widner W), X55416 (Dankocsik C), AF164666 (Chen), AF336115 (Li C), AY297091 (Wang, Y), X57252 (Wu D). Nucleotide differences are indicated by squares.

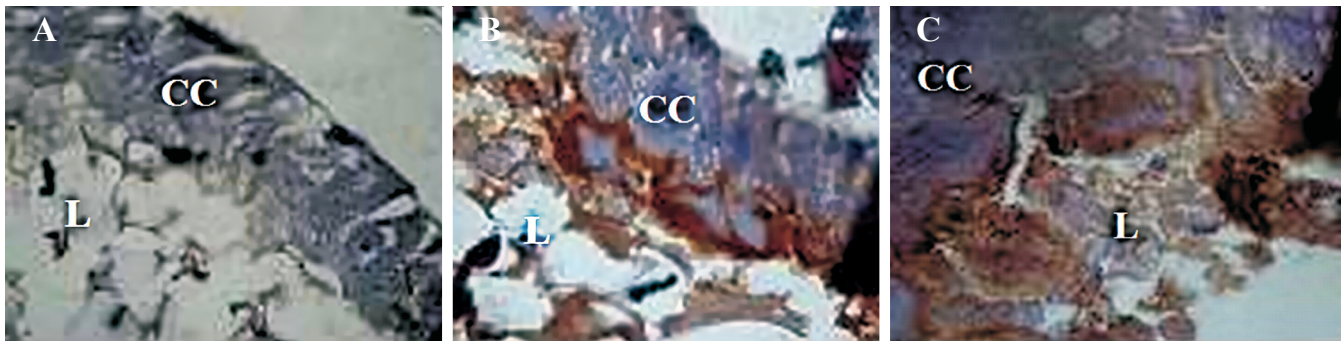


Figure 3. Immunolocalization of Cry proteins in the midgut of *T. solanivora* larvae fed with protoxins of 146-158-01 strain. (A) negative control; (B) anti-Cry1Ac; (C) anti-Cry2A. CC, columnar cells. L, intestinal lumen

more *et al.* 1998; Schnepf *et al.* 1998). Immunolocalization experiments showed that both Cry1Ac24 and Cry2Aa13, bind to the surface of midgut cells of *T. solanivora* larvae. The specific function of these proteins against this insect must be examined further but it could be possible that both proteins contribute to the toxicity. Combination of *cry1Ac* and *cry2Aa* genes could be a good alternative to control this insect pest and might to reduce the development of insect resistance against either Cry1Ac24 or Cry2Aa13 proteins.

Conclusions

Two native genes coding for Cry1Ac24 and Cry2Aa13 proteins have been isolated and characterized from a native *B. thuringiensis* strain with high activity against one of the most important potato pests in northern South America (*T. solanivora*). Some changes in amino acids could be responsible for the high activity against *T. solanivora*. Isolation and sequence analysis of these new *cry* genes increase the development of the use of Cry proteins as competitive biological insecticides. New isolation of *B. thuringiensis* in the future in conjunction with knowledge of their toxins will be essential in order to find optimal alternatives to control this insect, diminishing in this way problems associated with chemical insecticides.

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