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Susceptibility of *Anthonomus grandis* (Cotton Boll Weevil) and *Spodoptera frugiperda* (Fall Armyworm) to a Cry1Ia-type Toxin from a Brazilian *Bacillus thuringiensis* Strain

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Different isolates of the soil bacterium *Bacillus thuringiensis* produce multiple crystal (Cry) proteins toxic to a variety of insects, nematodes and protozoans. These insecticidal Cry toxins are known to be active against specific insect orders, being harmless to mammals, birds, amphibians, and reptiles. Due to these characteristics, genes encoding several Cry toxins have been engineered in order to be expressed by a variety of crop plants to control insect-pests. The cotton boll weevil, *Anthonomus grandis,* and the fall armyworm, *Spodoptera frugiperda,* are the major economically devastating pests of cotton crop in Brazil, causing severe losses, mainly due to their endophytic habit, which results in damages to the cotton boll and floral bud structures. A *cry1Ia*-type gene, designated *cry1Ia12*, was isolated and cloned from the *Bt* S811 strain. Nucleotide sequencing of the *cry1Ia12* gene revealed an open reading frame of 2160 bp, encoding a protein of 719 amino acid residues in length, with a predicted molecular mass of 81 kDa. The amino acid sequence of Cry1Ia12 is 99% identical to the known Cry1Ia proteins and differs from them only in one or two amino acid residues positioned along the three domains involved in the insecticidal activity of the toxin. The recombinant Cry1Ia12 protein, corresponding to the *cry1Ia12* gene expressed in *Escherichia coli* cells, showed moderate toxicity towards first instar larvae of both cotton boll weevil and fall armyworm. The highest concentration of the recombinant Cry1Ia12 tested to achieve the maximum toxicities against cotton boll weevil larvae and fall armyworm larvae were 230 μg/mL and 5 μg/mL, respectively. The herein demonstrated insecticidal activity of the recombinant Cry1Ia12 toxin against cotton boll weevil and fall armyworm larvae opens promising perspectives for the genetic engineering of cotton crop resistant to both these devastating pests in Brazil.

Keywords: *Anthonomus grandis*, *Bt* toxin, Coleoptera, Cotton, Cry1Ia, *E. coli* expression, Lepidoptera, *Spodoptera frugiperda*

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Introduction

Bacillus thuringiensis (*Bt*), a well-known entomopathogen, is a Gram-positive spore-forming bacterium, which forms parasporal crystal (Cry) protein inclusions during the stationary growth phase (Bravo *et al.*, 1998; Schnepf *et al.*, 1998). These crystal inclusions are produced by one or more insecticidal proteins, which can exhibit toxicity and specificity toward a select group of Lepidopteran, Coleopteran and Dipteran insect species. The crystals are solubilized by the alkaline conditions existing in the midgut lumen of susceptible insect larvae, releasing Cry protoxins, which are enzymatically processed and converted into active Cry toxins (de Maagd *et al.*, 2003). After activation the toxins interact with specific cell surface docking molecules and destroy the brush border membrane integrity by pore formation. These pores disrupt the osmotic regulation of the insect intestinal epithelium resulting in cell

swelling, eventual cell lysis and insect death (Hofmann *et al.*, 1988; Schwartz *et al.*, 1993; Knowles, 1994).

Microbial insecticide formulations based on *Bt* are used worldwide as biopesticides and different *cry* genes encoding toxic Cry proteins showed great potential in the control of several economically devastating insect-pests when bioengineered in crop plants (Betz *et al.*, 2000; Chattopadhyay *et al.*, 2004). To date, many plant species have been genetically modified with *cry* genes, resulting in transgenic plants with high level of resistance to insect pests (Hilder and Boulter, 1999; Van Rie, 2000; Christou *et al.*, 2006).

The cotton boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae), and the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), are devastating cotton pests responsible for more than 50% of insecticide costs in Brazilian cotton crop fields. Moreover, the endophytic habit of the *A. grandis* larvae inside floral buds results in destruction of the fiber quality, hampers the chemical control, causing considerable yield losses (Haynes *et al.*, 1992). The most promising cost-effective and sustainable method to control cotton boll weevil is the development of genetically resistant cotton lines that suppress the insect larval development.

Bacillus thuringiensis S811 is a novel Brazilian soil isolated strain, with toxicity against Lepidoptera and Coleoptera, including *S. frugiperda* and *A. grandis* insect-pests (Martins *et al.*, 2005a; Quezado, 2006). Among c.a. 40 Cry1-type proteins described to date in "The *Bacillus thuringiensis* Toxin Specificity Database" (http://www.glfc.cfs.nrcan.gc.ca/science/research/netintro99_e.html), the majority of them is active against Lepidoptera species, whereas few reports have shown that Cry1-type proteins are also toxic against Coleopteran species. Tailor *et al.* (1992) showed that the formally designated Cry5 toxin, presently designated as Cry1Ia1 toxin (Crickmore *et al.*, 1998), is toxic to both Lepidoptera and Coleoptera. In respect to *Spodoptera* species, *Spodoptera litura* has been shown to be susceptible to a Cry1Ia-type protein (Sasaki *et al.*, 1996).

Searching for novel sources of *cry* genes encoding new Cry toxins active against cotton insect-pests important within Brazil, a *cry1Ia*-type gene was isolated from the Brazilian S811 *Bt* strain. Herein, we report the cloning and characterization of the *cry1Ia12* gene, its expression in *E. coli* cells and also its insecticidal specificities and activities towards both *A. grandis* and *S. frugiperda* cotton insect-pests.

Materials and Methods

Bacteria strain. The *B. thuringiensis* S811 Brazilian strain obtained from Embrapa Genetic Resources and Biotechnology collection (*Bt* germoplasm bank, Brasília, Brazil- http://sicol.cria.org.br/crb/BGB) was used for to isolate the *cry1Ia12* gene. This strain was selected based on its toxicity towards cotton boll weevil (*A. grandis*), determined through selective bioassay (Quezado, 2006).

Isolation of a *cry1I* gene from the *B. thuringiensis* strain S811. *B. thuringiensis* strain S811 cells, grown in 30 mL of NYSM medium (0.8% nutritive broth, 0.1% yeast extract, 0.1% KH_2PO_4, 0.01% $CaCO_3$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 7H_2O$ and 0.001% $ZnSO_4 \cdot 7H_2O$, pH 7.0) for 12 h at 200 rpm and 30°C, were centrifuged and the pellets were kept at –20°C for DNA extraction. The CTAB (cationic hexadecyl trimethyl ammonium bromide) method (Doyle and Doyle, 1987) was used for total DNA isolation from the *B. thuringiensis* strain S811 cells. Polymerase Chain Reaction (PCR) using a general primer set to detect *cry1* genes was used to detect *cry1*-type genes within the *B. thuringiensis* strain S811 total DNA (Ceron *et al.*, 1995, Bravo *et al.*, 1998). In order to identify *cry1I*-type genes among the *cry1*-type genes detected in *B. thuringiensis* strain S811 total DNA, primers to amplify complete *cry1I*-type genes (i.e. from the start codon till the stop codon) were designed on the basis of multiple alignment of all previously described *cry1I* and the consensus sequences of their N- and C-terminal coding regions. PCR were performed using these primers designed to amplify complete *cry1I* genes (forward primer: 5'-ATGAAACTAAAGAATCAAGATAAG C-3', reverse primer 5'-CTAGATGTTACGCTCAATATGG-3'), 30 cycles of amplification at 42°C annealing temperature and high fidelity Taq DNA polymerase (Invitrogen). The resulting PCR fragment was excised from the gel and purified using the Geneclean® II Kit (Q-BIOGene), following the manufacturer's instruction.

The complete *cry1Ia12* gene sequence was amplified and cloned using specific forward 5'-ATGAAACTAAAGAATCAAGATAA GC-3' and reverse 5'-CTAGATGTTACGCTCAATATGG-3' PCR primers, designed on the basis of highly conserved N-terminal and C-terminal coding regions after alignment of all previously described *cry1I* sequences.

Cloning, sequencing and sequence analysis of the *cry1Ia12* gene from the *B. thuringiensis* strain S811. The purified PCR fragment putatively corresponding to a *B. thuringiensis* strain S811 *cry1I* gene was cloned into the pGEM-T Easy vector (Promega, Maldison, WI, USA) and used to transform *E. coli* XL1 Blue cells. The clone was sequenced using an ABI 3700 automated sequence analyzer (Applied Biosystem Perkin Elmer). Computer analyses of the cloned DNA sequence were done using the GCG software package (Genetics Computer Group, University of Wisconsin). Databank comparisons of the cloned DNA sequence with other published *cry* sequences were made using the BLASTx software from the NCBI databank (http://www.ncbi.nlm.nih.gov). The Conserved Domain Database search (CDD-search) from the NCBI site was used to compare motif identity and similarity with the clone DNA sequence and known conserved domains. Sequence alignments and dendrograms were obtained by using CLUSTAL W software and were edited with the BOXSHADE software (http://www.ch.embnet.org/software/BOX_form). Dendrograms were edited using the TreeView software (http://darwin.zoology.gla.ac.uk/~rpage/treeviewx). The molecular mass and predicted *pI* of the clone deduced protein sequences were determined by the Protein Machine software available at the EXPASY site (http://us.expasy.org/tools/). The clone reported here was identified as a novel *cry1Ia* gene from the *B. thuringiensis* strain S811 and was named *cry1Ia12*. The sequence of the *cry1Ia12* gene was submitted to the GeneBank, being AY788868 the assigned accession number.

Construction of *E. coli* expression vector pET101-*cry1Ia12*. *E. coli* XL1 Blue cells containing the *cry1Ia12* gene cloned into the pGEM-T Easy vector were grown in Luria-Bertani (LB) medium in the presence of 100 μg/mL ampicillin, for 14-16 h at 37°C and the plasmid DNA was isolated using the chloroform: isoamyl alcohol method (Sambrook *et al*., 2001). PCR was performed using the isolated plasmid as template, the forward primer 5'-CACCATGAA ACTAAAGAATCAAGATAAGC-3', the reverse primer 5'-TTCTG CCTCATATGTTACTTCTACC-3', 30 cycles of amplification at 50°C annealing temperature and high fidelity Taq DNA polymerase (Invitrogen). The resulting PCR fragment was excised from the gel and purified using the Geneclean® II Kit (Q-BIOGene), following the manufacturer's instruction. The purified PCR fragment was subcloned into the expression vector pET101 D-TOPO (Invitrogen), following the manufacturer's instructions. The resulting construction, encoding the recombinant *cry1Ia12* gene to be expressed fused to a C-terminal His-tag, was named pET101-*cry1Ia12*.

Recombinant Cry1Ia12 toxin expression and purification. *E. coli* BL21 Star (DE3) cells transformed with pET101-*cry1Ia12* were grown at 37°C in 2 L erlenmeyer flasks containing 500 mL of LB medium, at 200 rpm agitation, in the presence of 200 μg/mL ampicillin, until $O.D._{.600}$= 0.6-0.8. Cry1Ia12 expression was induced by addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) when an $O.D._{.600}$ = 0.7 was reached. Alternatively, the expression of recombinant His-tagged Cry1Ia12 was increased by cultivating the recombinant *E. coli* in a 5.0 L BBraun Biostat C bioreactor, and by using 20 g/L lactose for recombinant protein induction. Sixteen hours after induction the cells were harvested by centrifugation at 4,000 rpm. The pellet containing the cells expressing the Cry1Ia12 His-tagged protein was then resuspended in Lysis buffer (50 mM Sodium Phosphate buffer, 300 mM NaCl, 1% Glycerol and 0.5% Triton X-100, pH 7.0). The crude extract was sonicated three times for 5 min (large tip, Virsonic Cell Disrupter -Model 16-850), centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was analyzed in a 12% SDS-PAGE (Laemmli *et al*., 1970). The supernatant was also used for the partial purification of the recombinant Cry1Ia12 using Ni^{2+} nitrilo-triacetic acid affinity resin (Ni-NTA, QIAGEN) equilibrated with Equilibration buffer (50 mM Sodium Phosphate buffer, 300 mM NaCl, 10 mM Imidazole pH 7.0). The supernatant mentioned, containing the *E. coli* expressed recombinant Cry1Ia12 His-tagged protein, was incubated for 30 min within the equilibrated column. The column was then washed with two different buffers: first with 100 mL of Equilibration buffer and then 100 mL of the same buffer containing 2 mM Imidazole. The recombinant Cry1Ia12 His-tagged protein was eluted with 6 mL of Equilibration buffer containing 250 mM of Imidazole. All the steps were done using a flow rate of 2 mL/min. The eluted protein was dialyzed against 50 mM sodium carbonate buffer pH 10.5 and stored at 4°C. The purified protein was quantified according to the Lowry method (Lowry *et al*., 1951).

Bioassays of the recombinant Cry1Ia12 toxicity against insect larvae. Bioassays of the recombinant Cry1Ia12 toxicity against cotton boll weevil larvae were carried out in 40 mL of sterilized artificial diet as described by Monnerat *et al.* (2000). The recombinant Cry1Ia12 protein, purified and dialyzed as explained before, was incorporated in the diet at final concentrations of 50 μg/mL, 100 μg/mL and 230 μg/mL, respectively. The diet was added to Petri dishes and neonate larvae were placed in pits created in the artificial diet. After 7 days of incubation at 28°C ± 2, 55% ± 5 relative humidity and photoperiod of 14 h, the dead larvae were counted. In the negative control treatment, distilled water and dialysis buffer (50 mM sodium carbonate buffer pH 10.5) were added to the artificial diet. Each treatment was repeated four times and each replicate contained 15 larvae. For *S. frugiperda*, freshly prepared artificial diet, free of sorbic acid, nipagin and formaldehyde, at 50°C, was poured into a 24-well cell culture plate. After solidification, the recombinant Cry1Ia12 protein, purified and dialyzed, was applied on the diet surface in each well and allowed to dry. Subsequently, an 1-day-old second instar larva of *S. frugiperda* was added to each well. In the negative control treatment, distilled water and dialysis buffer (50 mM sodium carbonate buffer pH 10.5) were added to the artificial diet. Twenty-four larvae and one negative control were tested. The plates were covered with acrylic lids and incubated at the same conditions used for rearing the insects. After 48 h, the surviving larvae were individually transferred to 50 mL cups containing rearing diet and the mortality was assessed. Larval mortality was assessed again at day 5 (Praça *et al.*, 2004). To calculate the mortality value, decimal serial dilutions of the protein were made. Five dilutions of the protein and 24 larvae per dilution were tested, following the same procedure above mentioned. The bioassays were repeated three times with twenty-four larvae and one negative control. Mortality data were analyzed and calculated.

Results

Cloning and sequence analyses of the *cry1Ia12* gene. PCR of *cry1*-type genes using a set general primers resulted in amplification of a 0.3-kb fragment, indicating the presence of a *cry1*-type gene in the *B. thuringiensis* S811 strain (data not shown). Further PCR with primers specifically designed to detect *cry1I*-type genes, among the previously detected *cry1*-type genes found within the genome of the *B. thuringiensis* S811 strain, resulted in the amplification of a fragment of approximately 2200 bp (data not shown). This putative *cry1I*-type gene was cloned into the vector pGEMT-easy, and sequencing analysis revealed a high nucleotide sequence similarity of the insert with *cry1Ia* sequences previously reported (data not shown). This sequence was designated *cry1Ia12* gene, since other 11 *cry1Ia* genes (*cry1Ia1* till *cry1Ia11*) are already described in public sequence databases. The *cry1Ia12* gene comprised a 2160 bp open reading frame encoding a predicted protein of 719 amino acid residues (Fig. 1) and predicted *pI* value of 6,21. Amino acid sequence homology analyses among ten known holotypes Cry1Ia proteins indicated that the sequence of the deduced amino acid sequence of the *cry1Ia12* gene (i.e. Cry1Ia12 protein) is 99% identical to the amino acid sequences of Cry1Ia1, Cry1Ia2, Cry1Ia3, Cry1Ia4, Cry1Ia5, Cry1Ia6, Cry1Ia8, Cry1Ia10 and Cry1Ia11. The amino acid sequence of Cry1Ia12 is 96% identical to the amino acid sequence of Cry1Ia7. Concerning the amino acid sequence of other Cry1I-type proteins, the

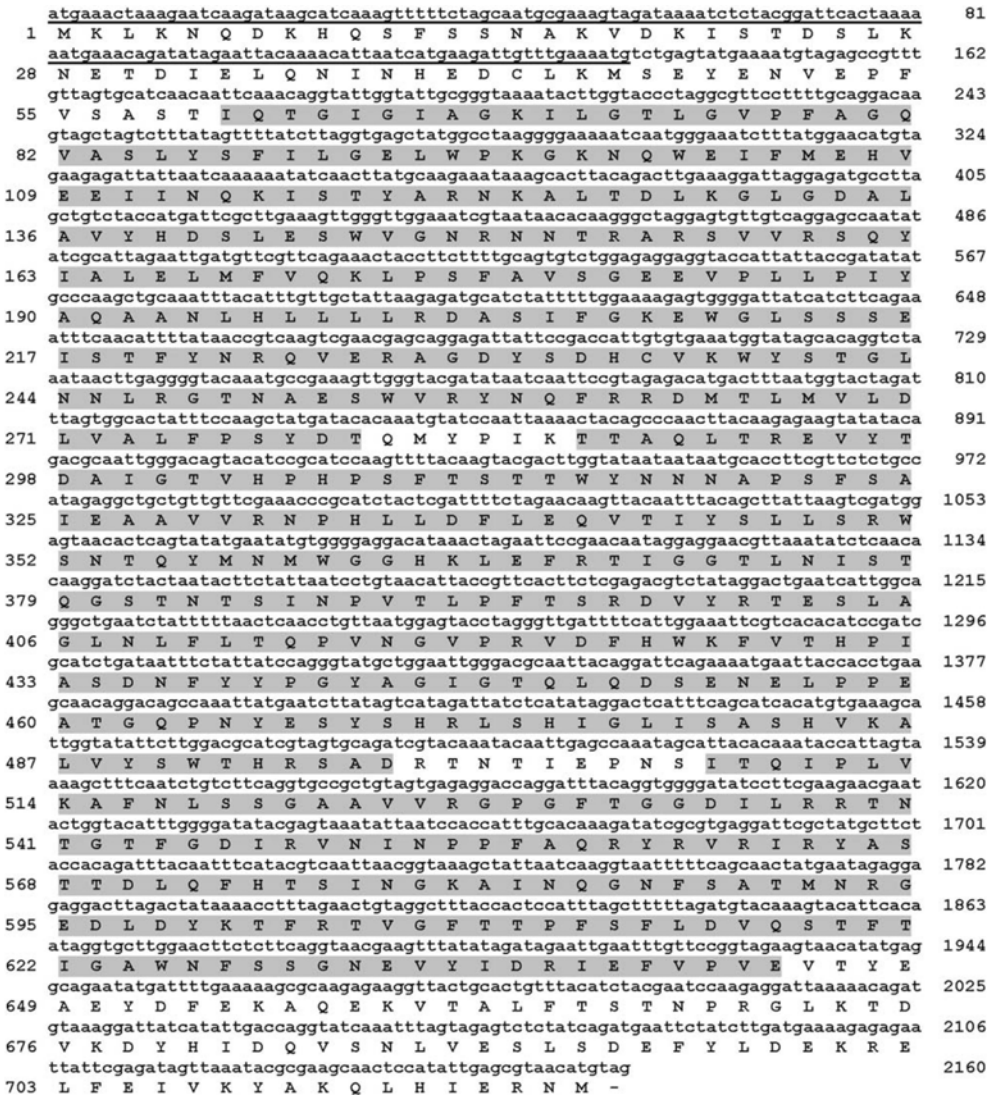

Fig. 1. Nucleotide sequence of the *cry1Ia12* gene (GenBank accession number AY788868) and the deduced amino acid sequence of the corresponding Cry1Ia12 protein. The N-terminal sequence has been underlined. The three classic structural domains present in Cry proteins are in boldface and shaded.

amino acid sequence of Cry1Ia12 is 93% identical to Cry1Ie1, 92% identical to Cry1Ib1 and 89% identical to Cry1Ic1, Cry1Ic2 and Cry1Id1. Amino acid sequence alignment among Cry1I-type proteins revealed that Cry1Ia12 contains amino acid residues, which are divergent in relation to the other Cry1I-type proteins along all its three structural domains (Fig. 2). When compared with the others *cry1Ia*-type genes, *cry1Ia12* exhibits substitutions at positions 476 (a for g) and 1719 (c for t), resulting in a single replacement of the Lys^{159} by Arg at domain I (located at the second alpha-helix) (Fig. 1 and 2). This replacement also occurs in the *cry1Ia5* sequence (Selvapandiyan *et al.*, 1998) in this exact position. Compared to *cry1Ia1* (Tailor *et al.*, 1992) and *cry1Ia3* (Shin *et al.*, 1995) there was a substitution at position 697 (t for g), resulting in the replacement of the Tyr^{233} by Asp at domain I. Compared to the *cry1Ia3*, *cry1Ia11* and *cry1Ia5* genes there were substitutions in the *cry1Ia12* sequence at positions 2133 (c for g) and 2134 (g for c), corresponding to the replacement of the Asn^{711} by Lys and of the Glu^{712} by Gln, both at domain III, respectively (Fig. 1 and 2). Cry1Ia7 represents an exception, since it is the most heterologous Cry1Ia-type protein, differing from Cry1Ia12 in 28 amino acid residues, mainly localized within the Domain II (Fig. 2). Among all the Cry1I-type proteins aligned with Cry1Ia12, the domain I is the most heterologous while the domain III is the most conserved one (Fig. 2).

Recombinant Cry1Ia12: *E. coli* expression, purification and evaluation of toxicity against insect larvae. The *E. coli* BL21 Star (DE3) strain harboring the pET101-*cry1Ia12* construct

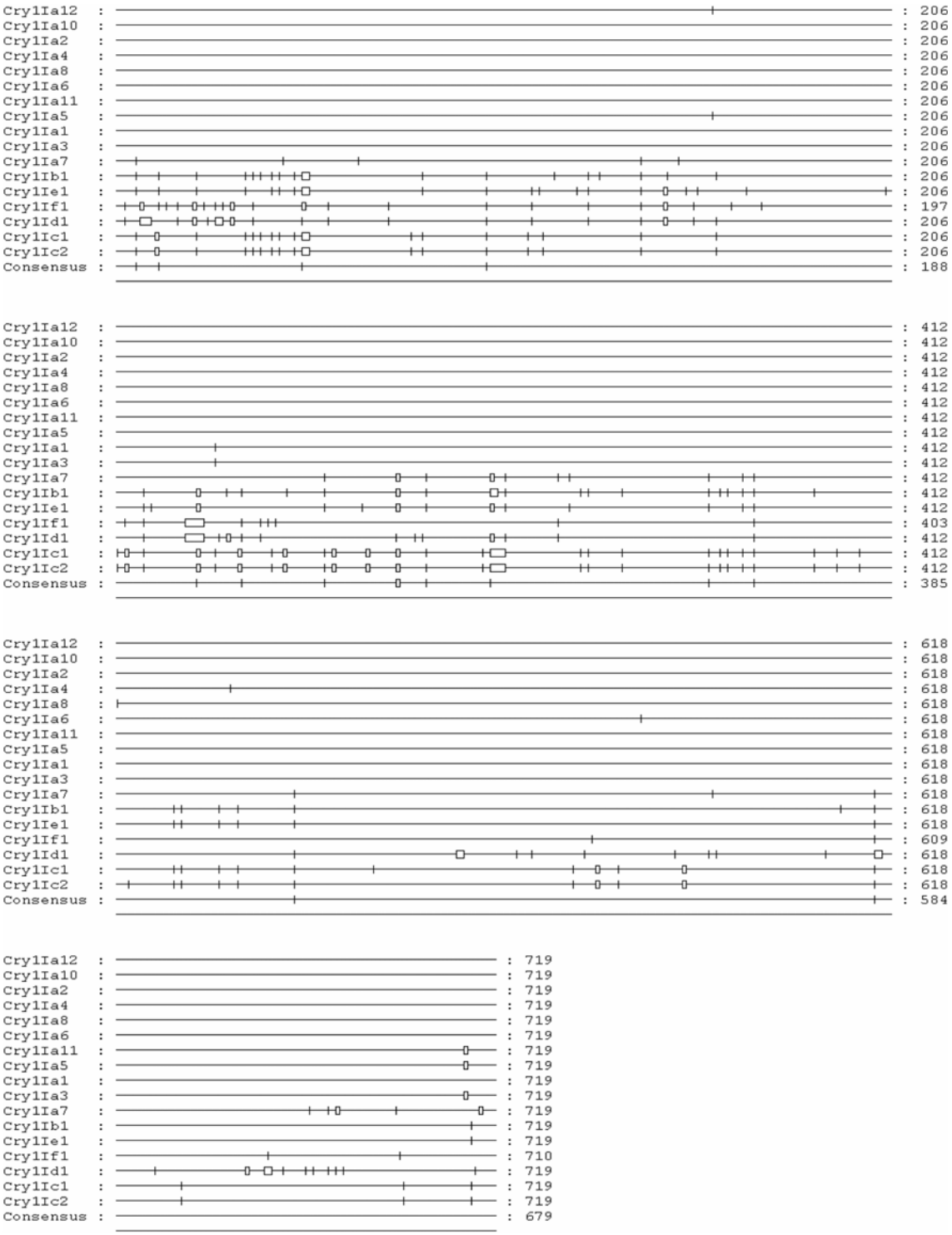

Fig. 2. Alignment comparison among the amino acid sequences of Cry1Ia12 and other Cry1I-type proteins. The horizontal lines represent the conserved amino acid sequence stretches among the proteins. Vertical lines and rectangles represent single amino acids and amino acid sequence stretches, respectively, which are divergent among the proteins. The three structural domains in Cry1Ia12 protein sequence are at positions 60 to 282 (Domain I), 287 to 487 (Domain II) and 507 to 644 (Domain III).

was induced with 1 mM IPTG to express the recombinant His-tagged Cry1Ia12 protein. SDS-PAGE analysis of *E. coli* extracts after IPTG induction, showed a differential protein band corresponding to the expected 74-kDa recombinant His-

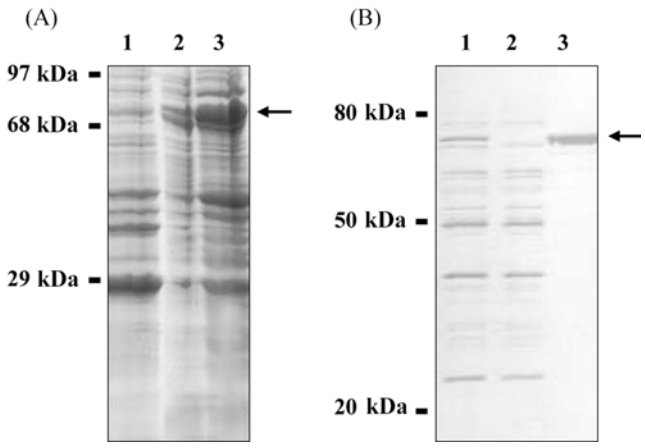

Fig. 3. (A) SDS-PAGE (12%) analysis showing the expressed recombinant Cry1Ia12 protein. Lane 1. Non-induced *E. coli* strain BL21Star (DE) containing the expression vector without insert was used as a negative control of the expression. Lane 2. *E. coli* crude extract induced with 1 mM IPTG after 6 h expressing recombinant Cry1Ia12 protein, sonicated and centrifuged. Lane 3. *E. coli* crude extract induced with 1 mM IPTG after 12 h expressing recombinant Cry1Ia12 protein, sonicated and centrifuged. Arrows indicate the expressed 74-kDa recombinant Cry1Ia12. (B) SDS-PAGE (12%) showing the purified recombinant Cry1Ia12 protein. Lane 1. *E. coli* crude extract inducted with 1 mM IPTG after 12 h expressing the recombinant Cry1Ia12 protein. Lane 2. Fraction of *E. coli* crude extract (from Lane 1) which was not retained in the columm. Lane 3. Recombinant Cry1Ia12 protein purified by using Ni-NTA column. Arrow shows the expected purified 74-kDa recombinant Cry1Ia12 protein.

tagged Cry1Ia12 protein (Fig. 3A). No additional bands were identified within extracts of non-induced cells with empty pET101 vector (Fig. 3A). The expression conditions were optimized to increase the yield of recombinant His-tagged Cry1Ia12 by cultivating the recombinant *E. coli* in 5 L bioreactor and by using 20 g/L lactose induction. In these conditions, a large amount of the recombinant His-tagged Cry1Ia12 protein was produced and, subsequently, purified using Ni^{2+}-NTA affinity chromatography from sonicated *E. coli* crude extract (Fig. 3B). The toxicity of purified recombinant His-tagged Cry1Ia12 was tested in diet bioassays against cotton boll weevil and fall armyworm larvae. The bioassays revealed that both insect pest larvae were susceptible to the purified recombinant His-tagged Cry1Ia12 (Fig. 4). The purified recombinant His-tagged Cry1Ia12 was toxic to *A. grandis* and to *S. frugiperda* larvae, being 230 μg/mL and 5 μg/mL the highest concentrations tested to achieve the maximum toxicities, respectively (Fig. 4). Additional bioassays against the same insect larvae, using different recombinant Cry proteins, including Cry3Aa, Cry1Aa, Cry1Ac, and Cry1Ba were performed (data not shown). Except for Cry1Ia12 toxin, no other tested Cry protein was significantly toxic to *A. grandis*, and no other tested Cry toxin had effect on larval growth or mortality rates.

Discussion

Here we report the isolation of a *cry1Ia*-type gene from the Brazilian *Bt* strain S811, named c*ry1Ia12* gene, which presents marginal insecticidal activity against *A. grandis* and reasonable toxicity against *S. frugiperda*. The Cry1Ia12 protein sequence differs from the other Cry1Ia-type proteins only in one or two amino acid residues localized along the three structural domains (I, II and III) involved with the insecticidal activity of the Cry proteins. Cry1Ia7 is the most heterologous *cry1Ia*-type gene, differing from Cry1Ia12 in 28 amino acid residues, mainly localized within the Domain II. Considering all the

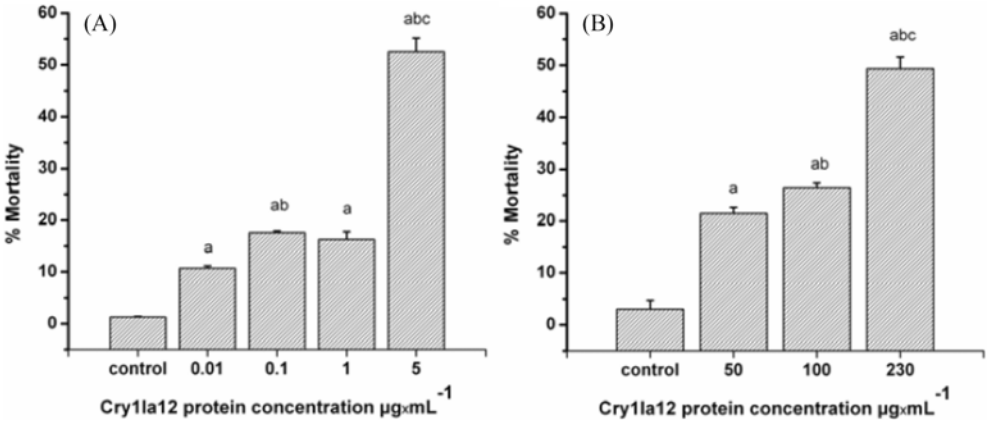

Fig. 4. Bioassays of purified recombinant His-tagged Cry1Ia12 toxicity against cotton insect-pests. All bioassays were conducted three times with different amounts of recombinant Cry1Ia12 protein. a, b, c: means significant differences determined by the Tukey test ($p < 0.05$). In the graphics, (a) differs from the controls at 0.05% and (c) differs from (a) and (b) at 0.05%. (A) Bioassay of purified recombinant His-tagged Cry1Ia12 toxicity against *S. frugiperda*. Four concentrations of recombinant Cry1Ia12 were tested: 0.010, 0.100, 1 and 5 μg/mL. (B) Bioassay of purified recombinant His-tagged Cry1Ia12 toxicity against *A. grandis*. Three concentrations of recombinant Cry1Ia12 were tested: 50, 100 and 230 μg/mL.

Table 1. Insecticidal activity of various Cry1I-type toxins, previously described in the literature

Insect			Cry1I-type toxin	Assay type (a)	LC_{50} (d)	Active (f)	Reference (g)
Order	Scientific name	Common name					
Coleoptera	*Agelastica coerulea*	alder leaf beetle	Cry1Ia3	LD	>1900 µg/mL	N	Shin *et al.*, 1995
			Cry1Ia3	LD	>1900 µg/mL	N	Choi *et al.*, 2000
			Cry1Ib1	LD	>2600 µg/mL	N	Shin *et al.*, 1995
			Cry1Id1	LD	>960 µg/mL	N	Choi *et al.*, 2000
	Diabrotica spp.	corn rootworm	Cry1Ia4	dns	dns	N	Kostichka *et al.*, 1996
	Leptinotarsa decemlineata	Colorado potato beetle	Cry1Ia1	LD	250 µg/mL	Y	Tailor *et al.*, 1992
			Cry1Ia4	dns	dns	N	Kostichka *et al.*, 1996
	Phaedon brassicae	daikon leaf beetle	Cry1Ia3	LD	>1900 µg/mL	N	Shin *et al.*, 1995
			Cry1Ib1	LD	>2600 µg/mL	N	Shin *et al.*, 1995
	Pyrrhalta aenescens	elm leaf beetle	Cry1Ie1	LD	dns	N	Song, *et al.*, 2003
	Tenebrio molitor	yellow mealworm	Cry1Ia2	DI	>2000 µg/mL	N	Gleave *et al.*, 1993
Diptera	*Culex pervigilans*	mosquito	Cry1Ia2	FI	>2000 µg/mL	N	Gleave *et al.*, 1993
Lepidoptera	*Artogeia rapae*	imported cabbageworm	Cry1Ia3	LD	0.11 µg/cm^2	Y	Koo *et al.*, 1995
	Bombyx mori	domestic silkworm	Cry1Ia1	LD	0.10-1.00 µg/mL **(e)**	Y	Sasaki *et al.*, 1996
			Cry1Ia3	DI	10.90 µg/mL	Y	Shin *et al.*, 1995
			Cry1Ia3	DI	8.70 µg/cm^2	Y	Koo *et al.*, 1995
			Cry1Ia3	DI	7.08 µg/mL	Y	Choi *et al.*, 2000
			Cry1Ib1	DI	>260 µg/mL	N	Shin *et al.*, 1995
			Cry1Id1	DI	439.56 µg/mL	Y	Choi *et al.*, 2000
	Epiphyas postvittana	light brown apple moth	Cry1Ia2	DI	67 µg/mL	Y	Gleave *et al.*, 1993
	Helicoverpa armigera	cotton boll worm	Cry1Ia5	IP	dns	Y	Selvapandiyan *et al.*, 1998
			Cry1Ie1	DI	dns	N	Song, *et al.*, 2003
	Hyphantria cunea	fall webworm moth	Cry1Ia3	DI	> 46.32 µg/cm^2	N	Koo *et al.*, 1995
	Leguminivora glycinivorella	soybean pod borer	Cry1Ie1	LD	9.02 µg/mL	Y	Song, *et al.*, 2003
	Ostrinia furnacalis	Asian corn borer	Cry1Ie1	DI	2.22 µg/mL	Y	Song, *et al.*, 2003
			Cry1Ie1	DI **(b)**	6.58 µg/mL	Y	Liu *et al.*, 2004
			Cry1Ie1	DI **(c)**	dns	Y	Liu *et al.*, 2004
	Ostrinia nubilalis	European corn borer	Cry1Ia1	DI	16 µg/mL	Y	Tailor *et al.*, 1992
			Cry1Ia1	DI	3.34 µg/mL	Y	Sekar *et al.*, 1997
			Cry1Ia4	dns	dns	Y	Kostichka *et al.*, 1996
	Plutella xylostella	diamondback moth	Cry1Ia1	LD	0.001-0.01 µg/mL **(e)**	Y	Sasaki *et al.*, 1996
			Cry1Ia3	LD	17.40 µg/mL	Y	Shin *et al.*, 1995
			Cry1Ia3	LD	0.089 µg/cm^2	Y	Koo *et al.*, 1995
			Cry1Ia3	LD	12.90 µg/mL	Y	Tabashnik *et al.*, 1996
			Cry1Ia3	LD	2.57 µg/mL	Y	Choi *et al.*, 2000
			Cry1Ia4	dns	dns	Y	Kostichka *et al.*, 1996
			Cry1Ib1	LD	147.80 µg/mL	Y	Shin *et al.*, 1995
			Cry1Id1	LD	4.26 µg/mL	Y	Choi *et al.*, 2000
			Cry1Ie1	LD	0.20 µg/mL	Y	Song, *et al.*, 2003
	Spodoptera exigua	beet armyworm	Cry1Ia3	LD	> 66 µg/ cm^2	N	Koo *et al.*, 1995
			Cry1Ie1	DI	dns	N	Song, *et al.*, 2003
	Spodoptera litura	tobacco cutworm	Cry1Ia1	LD	0.1-1.0 µg/mL **(e)**	Y	Sasaki *et al.*, 1996

(a) LD = leaf dip (Tabashnik *et al.*, 1993); DI = diet incorporation (of Cry proteins expressed in *E. coli*, *Bacillus* or transgenic plant); FI = free ingestion; IP = *in planta*; dns = data not shown.
(b) Bioassay performed with the modified Cry1Ia4 protein (Cry1Ia4m), encoded by a gene modified to incorporate the preferential codon usage of plants, expressed in *E. coli*.
(c) Cry1Ia4m expressed in transgenic tobacco. DI assay performed as described in He *et al.* (2003) *Journal of Economic Entomology* 96, 935-940.
(d) LC_{50} = lethal concentration that causes 50% mortality of the insects. mg/mL = mg of Cry protein/mL solid diet (in case diet incorporation bioassay) or mg/mL = mg of Cry protein/mL solution (in case of leaf dip or free ingestion bioassays), unless otherwise stated. mg/cm^2 = mg of Cry protein/cm^2 solid diet surface (in case diet incorporation bioassay) or mg/cm^2 = mg of Cry protein/cm^2 leaf disk surface (in case of leaf dip bioassay). dns = data not shown.
(e) mg/mL = mg of crystal protein/mL of crystal-spore mixture.
(f) The parameter is mortality. Y = yes/active; N = no/not active.
(g) Choi *et al.* (2000) *Current Microbiology* 41, 65-69; Gleave *et al.* (1993) *Applied and Environmental Microbiology* 59, 1683-1687; Koo *et al.* (1995) *FEMS Microbiology Letters* 134, 159-164; Kostichka *et al.* (1996) *Journal of Bacteriology* 178, 2141-2144; Liu *et al.* (2004) *In Vitro Cellular & Developmental Biology-Animal* 40, 312-317; Sasaki *et al.* (1996) *Current Microbiology* 32, 195-200; Sekar *et al.* (1997) *Applied and Environmental Microbiology* 63, 2798-2801; Selvapandiyan *et al.* (1998) *Molecular Breeding* 4, 473-478; Shin *et al.* (1995) *Applied and Environmental Microbiology* 61, 2402-2407; Song *et al.* (2003) *Applied and Environmental Microbiology* 69, 5207-5211; Tabashnik *et al.* (1996) *Applied and Environmental Microbiology* 62, 2839-2844; Tailor *et al.* (1992) *Molecular Microbiology* 6, 1211-1217.

Cry1I-type proteins compared to Cry1Ia12, the domain I is the most heterologous among them while the domain III is the most conserved one. Domains II and III are believed to be involved in recognition and binding of Cry proteins to the specific receptors on the brush border apical membrane of insect midgut cells, while the domain I is involved in membrane insertion and pore formation through the insect gut epithelium (de Maagd *et al.*, 2001). An additional role in pore formation has been proposed for Domain III (de Maagd *et al.*, 2001). The differences in amino acid sequences found along the three domains of Cry1Ia12 as compared to other Cry1I-type proteins may reflect eventual differences in the insecticidal activity of these toxins in terms of specificity against target insects and level of toxicity.

The majority of the Cry1I-type proteins (i.e. Cry1Ia, b, c, d, e, and f), expressed either in homologous systems (i.e. *Bacillus*) or in heterologous systems (i.e. *E. coli*; transgenic plant), exhibit activity mostly against Lepidoptera and rarely against Coleoptera (Table 1). In view of the high homologies of the Cry1Ia12 amino acid sequence to other Cry1Ia-type proteins, its activity against the Coleopteran species *A. grandis* was somewhat unexpected. Recently, another Cry protein codified by a *cry1Ia* gene, isolated from another *B. thuringiensis* strain, was expressed in *baculovirus* system and also showed activity towards *A. grandis* and *S. frugiperda* (Martins, 2005b). Tailor *et al.* (1992) have also found out that the product of a *cry1I*-type gene was toxic to larvae of the Coleoptera *Leptinotarsa decimlineata* (Colorado potato beetle), besides presenting toxicity to the Lepidoptera *Ostrinia nubilalis*.

To date, around 150 Cry toxin genes have been described in "The *Bacillus thuringiensis* Toxin Specificity Database" (http://www.glfc.cfs.nrcan.gc.ca/science/research/netintro99_e.html), many of them with known ability to control a great variety of insect pests, including members of Lepidoptera, Coleoptera, Diptera and Hymenoptera orders, as well as nematodes (Schnepf *et al.*, 1998; Hilder and Boulter, 1999; de Maagd *et al.*, 2001; de Maagd *et al.*, 2003). The Cry3Aa1 and the *Bt* binary toxin Cry23A/Cry37 were reported to be toxic to *A. grandis* (Hernstadt *et al.*, 1986; Donovan and Slaney, 2000). Nevertheless, except for the herein reported Cry1Ia12 toxin, to our knowledge none of the around 40 different Cry1-type toxins described to date were demonstrated be toxic to *A. grandis*. The maximum concentration tested to achieve the maximum toxicity (230 μg/mL; showed in this report) of the recombinant Cry1Ia12 against *A. grandis* weevil was similar to the reported toxicity of Cry1Ia1 against the Colorado potato beetle larvae (250 μg/mL) (Tailor *et al.*, 1992), which reflects a moderate insecticidal activity under the tested conditions.

Since the first report from almost 20 years ago of a Cry protein toxic to *A. grandi*s (Herrnstadt *et al.*, 1986), no transgenic plant resistant to boll weevil was ever reported, although this Coleoptera insect-pest is economically important in cotton crop in different producer countries, being the most devastating cotton insect pest in Brazil. The commercially available *Bt* transgenic cotton event Bollgard II (Monsanto) expresses Cry1Ac/Cry1Ab and Cry2Aa toxins that confers mild resistance against *S. frugiperda* (Hamilton *et al.*, 2004), and no confer resistance towards *A. grandis*. Our finding shows that the recombinant Cry1Ia12 protein is moderately toxic to the cotton boll weevil, besides being toxic to cotton fall armyworm in bioassays; opens, thus, promising perspectives to obtain Cry transgenic cotton lines resistant to both these devastating cotton pests.

It has been reported that several insect pests have developed resistance against insecticidal Cry proteins (Tabashnik *et al.*, 1993, 1997; Ferre and Van Rie, 2002). To avoid the development of resistance by insects to *Bt* toxins, it is important to use stacked gene strategy, which consists of a combination of c*ry* genes and/or other genes encoding insecticidal proteins within the same transgenic crop (Ferre and Van Rye, 2002; Christou *et al.*, 2006). However, one requirement for the stacked gene strategy to be efficient is that the stacked Cry toxins bind to different receptors within the target insect gut epithelium. Estela *et al.* (2004) showed that Cry1Ac and Cry1Ab toxins use different epitopes for binding gut brush border membrane vesicles of the Lepidoptera *Helicoverpa armigera*, making these c*ry* genes appropriate to compose a stacked gene strategy for cotton insect resistance. Therefore, isolation of novel *cry* genes, such as the presently described *cry1Ia12* gene, encoding proteins with presumable distinct modes of action against target cotton insect pests are crucial for pest control within cotton crop.

It will be interesting to test the toxicity spectrum of Cry1Ia12 against Coleopteran and Lepidopteran other than *A. grandis* and *S. frugiperda*. Also, it is important to test its toxicity even against nematodes that attack the cotton crop. Studies on the effect of the microbial flora present in the midgut of target insect larvae (Broderick *et al.*, 2006) on the toxicity of Cry1Ia12, studies on midgut receptors for Cry1Ia12 and studies on sequence mutagenesis impacts on the toxicity of Cry1Ia12 would definitely contribute to elucidate why Cry1Ia12 is less toxic to cotton boll weevil larvae than it is to fall armyworm larvae. Moreover, the *cry1Ia12* can be used as a DNA shuffling parental gene to generate *cry* genes with optimized and enhanced activity against important cotton pests. In conclusion, the insecticidal activity of Cry1Ia12 against *A. grandis* and *S. frugiperda*, and potentially against other relevant cotton pests, may prove to be valuable for transgenic control strategies, supported by beforehand meticulous bio-safety studies (Andow *et al.*, 2006), in field conditions.

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