Increased activity of a hybrid Bt *toxin against* Spodoptera frugiperda *larvae from a maize field in Cuba*

RESEARCH

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ABSTRACT

Spodoptera frugiperda is the most important maize pest in Cuba. The activity of seven *Bacillus thuringiensis* Cry1 toxins (five wild-type and two domain-swapped) was investigated in bioassays against newly hatched larvae from adults collected in a Cuban maize field. The CryAAC (1Ac/1Ac/1Ca) hybrid showed the lowest effective dose (50% lethal dose, LD_{50}) of 288 ng/cm^2 contrasting with parental Cry1Ca and Cry1Ac, which were not lethal (LD_{50} > 3000). The wild type Cry1Fa was the second most active toxin (LD_{50} 565 ng/cm^2). Hybrid H04 (1Ab/1Ab/1Ca) was only weakly toxic (LD_{50} 1750 ng/cm^2). To our knowledge, this is the first report where the CryAAC hybrid toxin is tested against this insect pest.

Key words: Bacillus thuringiensis, Cry δ-endotoxins, domain-swapped hybrid toxins, CryAAC, *Spodoptera frugiperda*, bioassays

Biotecnología Aplicada 2006;23:236-239

RESUMEN

Actividad incrementada de una toxina híbrida de *Bt* contra larvas de *Spodoptera frugiperda* de un campo de maíz en Cuba. *Spodoptera frugiperda* es el insecto plaga más importante del maíz en Cuba. La actividad de siete toxinas Cry1 de *Bacillus thuringiensis* (cinco salvajes y dos híbridos ínterdominios) hacia larvas recién nacidas a partir de adultos colectados en un campo cubano de maíz fue investigada mediante bioensayos. La toxina híbrida CryAAC (1Ac/1Ac/1Ca) mostró la menor dosis efectiva (dosis letal para el 50%, LD_{50}) de 288 ng/cm^2 en contraste con las parentales Cry1Ca y Cry1Ac, las cuales no fueron letales (LD_{50} > 3000). La toxina salvaje Cry1Fa fue la segunda más activa (LD_{50} 565 ng/cm^2). El híbrido H04 (1Ab/1Ab/1Ca) fue solamente débilmente tóxico (LD_{50} 1750 ng/cm^2). Hasta donde conocemos, este es el primer reporte donde la toxina híbrida CryAAC es ensayada contra este insecto plaga.

Palabras claves: Bacillus thuringiensis, δ-endotoxinas Cry, híbridos ínterdominios, CryAAC, *Spodoptera frugiperda*, bioensayos

Introduction

The insecticidal bacterium *Bacillus thuringiensis* (*Bt*) has been widely used in agriculture for the control of pest insects in crops [1]. The main *Bt* toxic factor, Cry δ-endotoxins, is produced as crystal parasporal bodies that form a large family of proteins with a remarkable structural similarity but with a wide target diversity [2]. Cry1 class comprises several Cry toxins that are highly specific for lepidopteran pests, which kill them following a unique mode of action [3]. The most accepted model for the Cry1 toxic pathway in Lepidoptera is as follows: crystals, after they are ingested by a susceptible insect, become soluble in the alkaline and reducing environment of the midgut lumen as 130-135-kDa protoxins that instantly suffer extensive proteolysis mainly by midgut serine proteases (trypsin- and chymotrypsin-like) and produce a 58-60-kDa protease resistant core. Then, activated toxins pass the peritrophic matrix and bind to a specific receptor(s) on the membrane of the columnar cells, where further proteolysis could take place before being inserted on the membrane and there by opening lytic pores. Insects ultimately die by inanition and septicemia [4, 5].

To date, the tertiary structure of six Cry toxins has been reported [6, 7, 8, 9, 10, 11]. Cry proteins have three structural domains of approximately 200 residues each. Domain I is formed by a bundle of seven anti-parallel α-helixes where a central amphipatic helix (α-5) is surrounded by the others. The function of this domain has been associated with pore formation in lipid rafts and membrane vesicles [6, 7]. Domain II consists of three anti-parallel β-sheets disposed in a "Greek key" topology adopting a so-called β-prism conformation. Domain III is formed by two β-sheets in a β-sandwich structure with a "jelly roll" topology. Both domain II and III have been associated with the recognition and binding of a receptor in midgut cells [3]. In the case of Cry1-class toxins, several studies have confirmed the presence of specificity determinants for the target insect on domain III and its swapping between different Cry1 toxins has successfully expanded the host range of parental toxins [12, 13, 14, 15]. Domain III has also been associated with the regulation of pore activity [3].

Fall armyworm (*Spodoptera frugiperda* Smith and Abbot) is the main maize pest in Cuba. Each year, this voracious insect produces production losses of about 40% to Cuban maize growers [16]. Since chemical pesticides are unspecific, highly polluting, and expensive, Cuban farmers have been using the conventional spray of *B. thuringiensis* spore/crystal

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mixtures for the last 30 years [17]. However, the high-cost of production and low on-plant persistence of *Bt* formulations have greatly limited the use of this technology. Transgenic *Bt*-crops represent an alternative for insect-pest control without these drawbacks [18]. As a first step in obtaining the fall armyworm-resistant transgenic Cuban maize plants, an optimized procedure for the genetic transformation of the Cuban elite-variety FR-28 has been reported [19]. As susceptibility to Cry toxins may vary among populations of a given insect species [20, 21], it is of great interest to determine the effect of different *Bt* toxins on local populations of the fall armyworm before introducing the insect-resistant trait into Cuban maize cultivars.

Here, we investigated the insecticidal potency of five of the most common lepidopteran-active Cry1 toxins and two Cry1 domain-swapped hybrid toxins of *B. thuringiensis* against neonate larvae of *S. frugiperda* from adults collected in a Cuban maize plantation.

Materials and methods

Bt toxins. *Bt* toxins were prepared from seven different *Escherichia coli* clones. Cry1Ac, Cry1Ab, Cry1Ca and Cry1Da toxins were purified from clones ECE53, ECE54, ECE125, and ECE129, respectively. They were kindly provided by D.R. Zeigler (BGSC, Ohio, USA). Clones pMH21 and H04 expressing Cry1Fa and the swapped toxin (1Ab/1Ab/1Ca), respectively, were kindly given by R.A. de Maagd (PRI, Wageningen, The Netherlands). The CryAAC (1Ac/1Ac/1Ca) hybrid toxin was obtained from clone pHYAAC, kindly donated by D.J. Ellar (Univ. Cambridge, UK).

Protoxin expression in *E. coli*, extraction, solubilization and activation with trypsin was done as previously described by Bosch et al. [22]. Trypsin-activated toxins (50 ng) were subjected to a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-12% PAGE) according to Laemmli [23]. Gels were visualized with Coomassie brilliant blue staining.

Protein determination. The activated toxins were quantified by the Lowry method [24] using bovine serum albumin (BSA) as the standard.

Bioassays. Bioassays were performed using the diet-surface contaminating procedure. Toxin preparations were serially diluted in six different toxin concentrations and tested by using 24 neonate larvae per concentration. *S. frugiperda* neonate larvae were obtained from eggs laid in our laboratory by adults previously collected from a maize plantation in southern Havana, the largest maize plantation area in Cuba. Toxin samples (20µl) were applied uniformly into each well (Multiwell-24 plates) with an artificial nutritive preparation [25] and then allowed to dry. Each larva was placed onto the nutritive surface and reared at 30ºC with a photoperiod consisting of 16 h of light and 8 h of darkness (16L:8D). Mortality was assessed after 7 days, where the effective dose (50% lethal dose of the toxins, LD_{50}) and 95% confidence limits (95% CL) were calculated using PROBIT analysis of the results from three independent bioassay experiments [26].

Results and discussion

To determine the susceptibility of *S. frugiperda* from a Cuban maize field to the *B. thuringiensis* Cry toxins, we studied the toxicity of Cry1Ab, Cry1Ac, Cry1Ca, Cry1Da and Cry1Fa and domain-swapped Cry1 hybrid toxins H04 (1Ab/1Ab/1Ca) and CryAAC (1Ac/1Ac/1Ca) against neonate larvae. The toxins tested in bioassays belong to the Cry1 class, which is active against lepidopteran larvae, though each toxin has a limited host range even within this insect order. Hybrid toxins H04 and CryAAC have been reported to be highly potent against beet armyworm, *Spodoptera exigua* H [14]. However, similar studies are not available for *S. frugiperda*.

Insect bioassays were conducted with activated toxins to simulate their presence in transgenic plants, since protoxins are not readily expressed by the plant cell machinery [27]. Results of SDS-12% PAGE analysis of *E. coli* purified wild-type and hybrid Cry1 toxins after trypsin proteolysis are shown in Figure 1. Trypsin activation yields products of expected sizes ranging from ~58 kDa (Cry1Ab and Cry1Ac) to ~60 KDa (Cry1Ca, Cry1Fa, Cry1Da, H04 and CryAAC).

Results of the bioassays are shown in table 1. Cry1Fa and CryAAC were the most active among all wild type and swapped Cry hybrid toxins tested, producing a considerable mortality in neonate fall armyworm larvae. CryAAC (LD_{50} 288 ng/cm^2) almost doubled the toxicity of Cry1Fa (LD_{50} 565 ng/cm^2). The importance of Cry1Ca domain III to determine insecticidal activity against other species of the genus *Spodoptera* has been previously described [14]. In addition to Cry1Ab and Cry1Ac, other Cry1 toxins (Cry1Ba, Cry1Ea, and Cry1Fa) became more active against beet armyworm when their domains I and II where combined with (part of) domain III of Cry1Ca. The CryAAC hybrid dramatically increased the toxicity of parental toxins (Cry1Ac and Cry1Ca)

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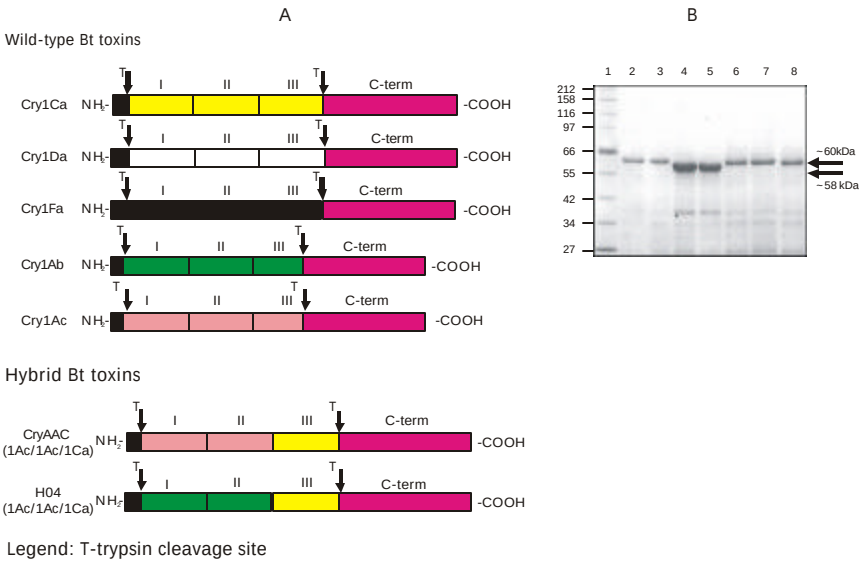

Figure 1. A schematic representation of the trypsin activation of *Bt* toxins used in this study A) and, SDS-PAGE profiles of trypsin-activated forms of recombinant *Bt* toxins produced in *E. coli* B). Lane 1, Broad-range molecular weight marker (NEB, USA); lane 2, CryAAC; lane 3, H04; lane 4, Cry1Ab; lane 5, Cry1Ac; lane 6, Cry1Ca; lane 7, Cry1Fa; lane 8, Cry1Da. Protein sizes in the marker are in kDa.

Table 1. Toxicities of trypsin-activated wild type and hybrid toxins administered to newborn *S. frugiperda* larvae

Toxin	LD50 (ng/cm2)	95% FL
Cry1Ac	> 3000	
Cry1Ab	> 3000	
Cry1Ca	> 3000	
Cry1Da	2430	(2010-2895)
Cry1Fa	565	(499-721)
H04	1750	(1200-2570)
CryAAC	288	(189-474)

against neonate *S. frugiperda*. Cry1Ca did not display enough mortality in bioassays for the estimation of LD_{50} but it showed a clear inhibitory effect on larval growth in the concentration range tested (data not shown). Cry1Ac did not have any effect on either mortality or larval growth. The rationale on how the combination of two toxins with low or no activity at all against *S. frugiperda* resulted in a hybrid toxin with an enhanced insecticidal activity, can not be formulated. A possible explanation could be found in the nature of *S. frugiperda* gut proteins to which these toxins bind. Previously, de Maagd et al. [28, 29] reported a greater toxicity of hybrid H04 against *S. exigua*. The authors considered that it had to do with an alteration in the binding sites of the toxin as determined in ligand blot assays using brush-border membrane vesicle (BBMV) preparations. Another explanation could be related to an improvement in pore activity. Luo et al. [30] found a high number of binding sites (B_{max} 5.4 pmol/mg BBMV) for Cry1Ac on *S. frugiperda* BBMV but a poor binding affinity (*Kd* 3 nM) and the incapacity of both altering membrane permeability (measured by a light scattering assay) and causing toxicity to neonate larvae in bioassays. Cry1Ca domain III substitution in Cry1Ac could improve the overall toxic properties against *S. frugiperda*. Thus, a more optimal combination of binding proteins recognized by a domain III hybrid could lead to improved toxicity by increasing the efficiency of membrane insertion and/or pore formation. More studies are needed to further characterize the mode of action of CryAAC.

Our study is the first report where CryAAC hybrid toxin is tested against *S. frugiperda*. A previous attempt of identifying effective *Bt* strains and Cry toxins against fall armyworm in Cuba was reported by Alfonso et al. [31]. These authors found that the inclusion proteins produced by *B. thuringiensis* var. *aizawai* strain 7.29 were the most toxic. It had been reported that this *Bt* strain produces at least three Cry1 δ-endotoxins of similar size but with different specificities [32]. Since authors used natural, purified and solubilized inclusions for bioassays they were probably unable to discriminate among the different strain 7.29 Cry1 toxins in orders find those responsible for the insecticidal activity against *S. frugiperda*. In this case, the high toxicity can be caused by a synergic interaction among the three Cry toxins produced by this strain.

The toxicity displayed by Cry1Fa is consistent with the findings of other authors, who described this toxin as highly active against fall armyworm [30], although they reported different values for LD_{50}. The variations of LD_{50} in different laboratories have been attributed to: a) the nature and mode of application of the toxins, b) the method used to estimate protein concentration, c) the temperature at which the bioassay is performed, and d) the origin of the insects [33].

We can therefore conclude that the neonate *S. frugiperda* larvae were highly susceptible to Cry1Fa and CryAAC toxins, while the latter was the most toxic. Cry1Da and swapped hybrid H04 were moderately toxic while Cry1Ac and Cry1Ab showed no toxicity. There was no mortality for Cry1Ca but a strong inhibitory effect was found on larvae growth at concentrations above 1 µg/cm^2. Hence, CryAAC and Cry1Fa can be used in transgenesis to confer resistance to *S. frugiperda* attack in crops such as maize and rice in Cuba. The present data, can not define whether CryAAC and Cry1Fa toxins can be deployed or not in a single transgenic event for insect-resistance management under field conditions. It would mainly depend on their ability of recognizing different receptors in the midgut cells of fall armyworm larvae. Future experiments will be focused on this interaction.

Acknowledgements

This study was supported by Grant 3031-243 of the Cuban Council of State. We would like to thank Ms. M. Ponce for her technical assistance.

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Received in may, 2006. Accepted for publication in october, 2006.