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Variant Cry1Ia toxins generated by DNA shuffling are active against sugarcane giant borer

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ABSTRACT

Sugarcane giant borer (*Telchin licus licus*) is a serious sugarcane pest in Americas whose endophytic lifestyle hampers effective chemical and biological controls. Therefore, development of alternative control methods is extremely important. Envisaging development of transgenic plants resistant to this pest, we investigated the effect of the *Bacillus thuringiensis* Cry protein Cry1Ia12synth (truncated protein lacking C-terminus with plant codon usage) and variants against *T. l. licus*. *cry1Ia12synth* gene was used to generate mutated variants, which were screened for toxicity toward *T. l. licus*. For that purpose, an innovative technique combining *cry* gene shuffling with phage-display was used to build a combinatorial library comprising 1.97×10^5 Cry1Ia12synth variants. Screening of this library for variants binding to *T. l. licus* Brush Border Midgut Vesicles led to the identification of hundreds of clones, out of which 30 were randomly chosen for toxicity testing. Bioassays revealed four variants exhibiting activity against *T. l. licus* as compared to the non-toxic Cry1Ia12synth. Eight single substitutions sites were found in these active variants. Based on theoretical molecular modelling, the probable implications of these mutations are discussed. Therefore, we have four genes encoding Cry1Ia12synth variants active against *T. l. licus* promising for future development of resistant transgenic sugarcane lines.

1. Introduction

Despite technological advances, sugarcane (*Saccharum officinarum* L.) culture still faces several phytosanitary problems and plant predators. Sugarcane giant borer, *Telchin licus licus* (Drury, 1773) (Lepidoptera: Castniidae), is one of the most important insect pests of the sugarcane crop, occurring in several countries of the Americas (Mendonça et al., 1996). The *T. l. licus* caterpillar develops inside the sugarcane stem, survives from one season to the next and causes significant annual economical losses (Mendonça, 1982). This endophytic lifestyle hampers the effectiveness of chemical, mechanical and biological control methods. The damage caused by this insect pest involves destruction of the basal internodes, reduction of sucrose, and formation of galleries that may compromise the entire diameter of the stem. This facilitates secondary infections by other insects and even more frequently by microorganisms (Mendonça et al., 1996). Therefore, the search for new alternatives for the control of this pest is of great importance for sugarcane producers.

In this context, a *Bacillus thuringiensis* (*Bt*) encoded entomotoxic protein (Cry) has been investigated for potential control of sugarcane giant borer neonate larvae. *Bt* is an aerobic, gram-positive bacterium that synthesizes crystalline inclusions during its sporulation that are composed of one or more Cry toxins and/or Cyt toxins (Höfte and Whiteley, 1989; Silva-Werneck and Ellar, 2008). Cry toxins are presently classified into 58 types (Cry1–Cry58) and many sub-types (e.g. Cry1Aa and Cry1Ba) based on their amino acid sequence similarity. They are active against a limited number of

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susceptible insect species (including lepidopterans, coleopterans and dipterans) and also against nematodes (Bravo and Soberón, 2008). A major group of Cry toxins is the three-domain (3D)-Cry family, members of which share similarities in sequence and structure. At least two different hypotheses have been proposed to explain the mode of action of these toxins, one relating to formation of pores in the target insect midgut and the other involving signal transduction. For both models, the first step is similar, i.e. the crystals are ingested by the larvae and solubilised in the midgut into protoxins. These are cleaved by midgut proteases to give rise to an active 60 kDa 3D-Cry toxin. The activated toxin binds to a cadherin receptor that is located in the midgut microvilli (Bravo and Soberón, 2008). The pore formation model suggests that this interaction with a cadherin receptor facilitates the proteolytic removal of the Cry α1-helix, triggering toxin oligomerisation that results in pore formation, causing larval death. In contrast, the signal transduction model proposes that binding of monomeric toxin to a cadherin receptor activates an adenylyl cyclase/protein kinase A magnesium-dependent signalling pathway, resulting in cell lysis (Bravo and Soberón, 2008; Zhang et al., 2006).

The spectrum of insects controlled by *Bt* can be broadened by directed molecular evolution techniques, such as DNA shuffling (Lassner and Bedbrook, 2001). DNA shuffling coupled with the phage-display technique has been valuable for the generation of genetic diversity and for selection of variants showing binding affinity to specific protein targets. DNA shuffling is an *in vitro* recombination method that uses small homologous DNA sequence fragments as substrates for PCR reactions, aiming to produce populations of gene variants (Stemmer, 1994; Zhao and Arnold, 1997). This technique has been used for several applications in different fields, including the generation of new molecules conferring resistance to insect pests (Patten et al., 1997). The phage-display approach involves the presentation of peptide and protein libraries on the surface of phage particles for facilitated selection of proteins with high affinity and specificity for a determined target (Willats, 2002).

In a previous study, it was reported that Cry1Ia12 protein exhibits considerable toxicity against the lepidopteran fall armyworm (*Spodoptera frugiperda*), indicating a potential for activity against other lepidopteran species (Grossi-de-Sa et al., 2007). Since the *cry1Ia12* gene was isolated by our research group and our bank of *cry* genes is still under construction, the *cry1Ia12* gene was therefore used to initiate the prospection and development of Cry toxins against *T. l. licus*. For this purpose, the nucleotide sequence of the original *cry1Ia12* gene was first changed to accommodate plant codon usage, resulting in *cry1Ia12synth*. Considering that Cry1Ia12synth is not toxic to *T. l. licus*, the aim of this work was to use the *cry1Ia12*synth gene to generate and select protein variants with toxicity towards *T. l. licus*. Then, DNA shuffling coupled with phage-display was used to generate a *cry1Ia12synth* combinatorial library. Screening of this combinatorial library for *cry1Ia12synth* variants that bind to Brush Border Midgut Vesicles (BBMVs) of *T. l. licus* resulted in the identification of new *cry1Ia12synth* molecules with entomotoxicity towards sugarcane giant borer larvae.

2. Materials and methods

2.1. Midgut dissection

T. l. licus second and third instar larvae, available in the field solely during part of sugarcane season, were collected in a sugarcane plantation in Maceió, AL, Brazil. The larval midguts were extracted and transferred to a microcentrifuge tube with MET buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris, pH 7.5) containing 1 mM PMSF. Then, midguts were centrifuged at 2500 × *g* for 5 min at 4 °C, the pellet was washed twice with MET buffer. The resulting pellet, enriched with midguts, was stored at −80 °C until use.

2.2. Preparation of brush border membrane vesicles (BBMVs)

Preparation of BBMVs from the dissected midguts of *T. l. licus* larvae was performed by using the precipitation method described by Wolfersberger et al. (1987). The concentration of BBMVs proteins was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as the standard for the calibration curve.

2.3. cry1Ia12synth gene

Previously, the *B. thuringiensis* S811 Brazilian strain, obtained from the collection of EMBRAPA Genetic Resources and Biotechnology, was used to isolate the *cry1Ia12* gene (Grossi-de-Sa et al., 2007) (GenBank accession no. AY788868). For the present work, the original *cry1Ia12* nucleotide sequence coding solely for the truncated toxin (lacking the C-terminus, i.e. consisting of the N-terminus and the domains I–III) was changed in order to accommodate plant genetic codon usage and named *cry1Ia12synth*. The *cry1Ia12synth* gene (1944 bp) was synthesized by Epoch Biolabs, Texas, US and cloned into the pBluescript II vector (Stratagene). The resulting sequence of *cry1Ia12synth* gene was deposited in the NCBI gene databank under accession number FJ938022.

2.4. DNA shuffling

First, the *cry1Ia12synth* gene was excised from the pBluescript II harbouring vector by digestion at 37 °C for 16 h under the following conditions: 5 μg plasmid DNA, 10 U *Not* I (Promega), 20 U *Sal* I (New England Biolabs), 1× Buffer D (Promega), 10 μg/mL BSA, in a 20 μL final volume. The digestion products were analysed by 1% agarose gel electrophoresis and the fragment corresponding to the *cry1Ia12synth* insert (1944 bp) was excised and purified from the agarose gel using the Geneclean II Kit (Bio 101). Then, 10 μg of the purified *cry1Ia12synth* gene was digested with 12.5 U of DNAse I (Invitrogen) at 15 °C for 15 min and then interrupted by addition of 5 μL of 0.5 M EDTA. Resulting fragments of 30–50 bp were jointly purified from a 2.5% agarose gel by using the High Pure PCR Product Purification Kit (Roche). Ten microlitres of the pool of purified fragments was used as template in a PCR without primers in a 25 μL final volume containing 0.4 mM dNTPs, 1 mM $MgSO_4$ and 2.5 U Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen), in 5× Platinum *Taq* DNA Polymerase High Fidelity buffer. The conditions of the primerless PCR were: 2 min 95 °C; 44 cycles: 1 min 95 °C, 1 min 42 °C and 1 min 72 °C (with a 5 s increase in extension time per cycle); with a final step of extension for 7 min at 72 °C. The products of the primerless PCR (1.5 μL) were used as template for a second PCR, containing the forward primer Cry1Ia12synthFOR (5′-CCCGGCCCAGGCGGCCATGAAACTCAAGAAC-3′) and the reverse primer Cry1Ia12synthREV (5′-CCGGCCGGCCTGGCCTTCGTAAGTAACTTC-3′). Both primers encode an *Sfi* I site, which is adequate for later cloning into the pCOMB3X phagemid (Andris-Widhopf et al., 2000). The second PCR, performed in a 100 μL final volume, contained 0.2 mM dNTPs, 2 mM $MgSO_4$, 0.8 μM each primer, 5 U *Taq* DNA Polymerase (Invitrogen) and 5 U Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen), in 1× Platinum *Taq* Buffer. The conditions for the second PCR were: 2 min 95 °C; 10 cycles: 30 s 95 °C, 1 min 42 °C and 1 min 72 °C; followed by 14 cycles: 1 min 95 °C, 1 min 42 °C and 1 min 72 °C (with 20 s increase in extension time per cycle); with a final step of extension for 10 min at 72 °C. The second PCR product corresponding to the DNA shuffling product was analysed by 1% agarose gel electrophoresis