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Midgut GPI-anchored proteins with alkaline phosphatase activity from the cotton boll weevil (*Anthonomus grandis*) are putative receptors for the Cry1B protein of *Bacillus thuringiensis*

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ABSTRACT

Cry toxins from *Bacillus thuringiensis* (Bt) are used for insect control. They interact with specific receptors located on the host cell surface and are activated by host proteases following receptor binding resulting in midgut epithelial cells lysis. In this work we had cloned, sequenced and expressed a *cry1Ba* toxin gene from the *B thuringiensis* S601 strain which was previously shown to be toxic to *Anthonomus grandis*, a cotton pest. The Cry1Ba6 protein expressed in an acrystaliferous *B. thuringiensis* strain was toxic to *A. grandis* in bioassays. The binding of Cry1Ba6 toxin to proteins located in the midgut brush border membrane of *A. grandis* was analyzed and we found that Cry1Ba6 binds to two proteins (62 and 65 kDa) that showed alkaline phosphatase (ALP) activity. This work is the first report that shows the localization of Cry toxin receptors in the midgut cells of *A. grandis*.

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1. Introduction

Bacillus thuringiensis (Bt) is an aerobic, spore-forming bacterium from the *Bacillus cereus* group that is distinguished due to its ability to produce Cry proteins with insecticidal activity. Cry proteins are produced as crystalline inclusions during the sporulation phase of growth (Feitelson et al., 1992; Bravo et al., 2005). To date, more than 400 *cry* genes have been sequenced and Cry proteins are classified into at least 55 groups organized into subgroups according to their amino acid sequence identities (Crickmore et al., 1998; Bt toxin nomenclature website at http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/).

Cry protein crystals are ingested and solubilized in the alkaline larval midgut. The solubilized protoxins are then activated by endogenous proteases. The active toxin crosses the peritrophic membrane and binds to specific receptors located in the brush border membrane of midgut columnar cells and eventually leads to cell death. As a final consequence, the larvae stop feeding and die by starvation or septicemia (Knowles, 1994; Schnepf et al., 1998; Sauka et al., 2007). The events that take place after binding are not yet clear, although the permeabilization of the membrane induced by toxin insertion and pore formation has long been proposed and there is much evidence that supports this view (Rodrigo-Simón et al., 2008; Bravo et al., 2007; Pigott and Ellar, 2007; Schnepf et al., 1998).

In a proposed model for the action of Cry1A toxins, cadherin-like and glycosyl phosphatidyl-inositol (GPI)-anchored receptors such as aminopeptidase N (APN) or alkaline phosphatase (ALP) has been shown to be crucial in several insects (Bravo et al., 2004; Pigott and Ellar, 2007). Cry1A toxins are proposed to bind to cadherin first, and then, after proteolytical modification of the bound toxin, a homo-oligomer is formed, which binds APN or ALP and inserts itself into the membrane of the columnar cell (Bravo et al., 2007). A recent model has challenged the pore-forming model and proposes that only binding to cadherin is required to produce cell death; binding to APN or ALP is irrelevant in this model, and pore formation may be but a secondary effect of the toxic events triggered by toxin binding to cadherin (Zhang et al., 2005, 2006).

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APN and ALP have been described as GPI-anchored receptors proteins for Cry1 toxins (Jurat-Fuentes and Adang, 2004; Crickmore and Ellar, 1992). In *H. virescens*, ALP is a 68-kDa GPI-anchored membrane glycoprotein (Jurat-Fuentes and Adang, 2004) and in *M. sexta* (Chen et al., 2005) and *A. aegypti* (Fernández et al., 2006), this protein is a 65-kDa GPI-anchored protein.

B. thuringiensis is used worldwide as a biopesticide controlling several lepidopteran, coleopteran, and dipteran insect pests (Schnepf et al., 1998). The most known toxins usually described as active against insects of the order Coleoptera are those of the Cry3 and Cry8 classes (Bravo et al., 1998; Abad et al., 2001), but other toxins from Cry1, Cry22, Cry34 and Cry35 classes have also been described as toxic to coleopteran insects (Baum et al., 2004; Baum and Light Mettus, 2000; Tailor et al., 1992).

Cotton boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), is the main pest of cotton in the South America (Busoli et al., 1994; Gallo et al., 2002). In order to decrease damage and loss of production, this pest has been controlled primarily by the use of chemical agents. However, while this has produced efficient cotton weevil control (Martin et al., 1987; Wolfenberger et al., 1997), these chemicals are expensive and may have negative environmental impacts. Other control methods employed are the use of pheromones (Gallo et al., 2002), early budding varieties, gathering and destruction of fallen flower buds (Silvie et al., 2001), biological control (Pallini et al., 2006) and integrated pest management (Luttrell et al., 1994). In addition, there is a possibility of producing transgenic cotton containing one or more *cry* genes to make the plant resistant to insect attack. On the other hand, to maintain the efficacy and to prevent the resistance of target species to transgenic Bt crops, the identity of putative Cry protein receptors and the interactions of Cry toxins with these receptors must be elucidated. Until now no Cry protein receptor has been identified in *A. grandis*.

In 2007, Martins et al. showed that the *B. thuringiensis* S601 strain (Embrapa, Genetic Resources and Biotechnology – Monnerat et al., 2001) was shown to be relatively toxic to a coleopteran insect (*A. grandis*) when compared to other coleopteran-specific Cry strains and a *cry1B* gene was identified in this strain. The S601 strain showed a LC_{50} of 140 μg/mL and other strains, like S1806, S1122 (*Bt* subsp. *tenebrionis*) and S1189 (*Bt* subsp. *israelensis*) showed LC_{50} of 300 μg/mL, 320 μg/mL and 740 μg/mL respectively. In this work we cloned the *cry1B* gene found in S601 strain, expressed its encoded protein in an acrystalliferous Bt strain, showed that the recombinant protein was toxic to *A. grandis* and detected *A. grandis* midgut proteins that bound toxin.

2. Materials and methods

2.1. Bacillus thuringiensis strains

In this work we used the acrystalliferous *B thuringiensis* subsp. *israelensis* IPS78/11 (Ward and Ellar, 1983) for the expression of the cloned *cry1Ba* gene, the S601strain (Entomopathogenic *Bacillus* strain collection at Embrapa Genetic Resources and Biotechnology – Brazil) that harbours the *cry1Ba6* gene and was used for bioassays and the *B. thuringiensis* subsp. *tenebrionis* T08 017 (S1122) (from the Collection of *B. thuringiensis* and *B. sphaericus* at the Institut Pasteur, Paris) as a reference of coleopteran-active isolate (Hofte et al., 1987).

2.2. Amplification, cloning expression of the cry1Ba gene

Molecular cloning techniques employed in this work were performed as described in Sambrook et al. (2001). All oligonucleotides used in this work were purchased from Invitrogen. All amplifications were performed by Platinum Taq DNA Polymerase High Fidelity (Invitrogen®). The *cry1Ba* gene of *B. thuringiensis* S601 strain, was amplified by PCR using the oligonucleotides Cry1BF (5×-**ggatcc**ATGacttcaaatagg-3′) and Cry1BR (5×-**ggatcc**ctattctt ccatgagaga-3′), which were designed from the published *cry1Ba* gene sequence (Genbank accession number: X06711) (Brizzard and Whiteley, 1988). *Bam*HI restriction sites were added to the sequence (letters in bold). The PCR program is available upon request. The amplified fragment was cloned into the vector pGEM-T easy (Promega) according to the manufacturer's instructions and sequenced (MEGA BACE 1000, Amersham Bioscience).

The plasmid containing the *cry1Ba*6 gene (pGEMCry1Ba6) was digested with *Bam*HI, and a 3687 bp fragment was separated by electrophoresis in an 0.8% agarose gel, eluted from the gel (Sephaglass™ Bandprep Kit, Pharmacia), according to the manufacturer's instructions. The purified fragment was cloned into the vector pSVP27A (Crickmore and Ellar, 1992; Crickmore et al., 1990), previously digested with *Bam*HI, producing the recombinant plasmid pSVP1B601 (not shown). One μg of DNA from the pSVP1B601 was used to transform an acrystalliferous *B thuringiensis* subsp. *israelensis* IPS78/11 by electroporation (1.8 kV, 400 Ω and 25 μF). The presence of the *cry1Ba*6 gene into the recombinant Bt was checked by PCR using the oligonucleotides described above.

The recombinant Bt Cry1Ba6 containing the *cry1Ba*6 gene was grown for 72 h in sporulation medium (Lereclus et al., 1995) supplemented with chloramphenicol (10 μg/mL) at 200 rpm and 30 °C until complete sporulation. Spores/crystals mixtures were washed three times with PBS (150 mM NaCl, 2.8 mM NaH_2PO_4, 4 mM $Na_2HPO_4 \cdot 7H_2O$, and pH 7.2) containing 1 mM EDTA, 0.1 mM PMSF, 1 μg/ml pepstatin and 5 μg/ml leupeptin. Crystals were isolated by centrifugation in sucrose gradients (Chang et al., 1993) and solubilized for 1 h at 4 °C with 0.1 M NaOH. The pH of the solution containing the solubilized protoxin was decreased to pH 9 by addition of same volume of 1 M Tris–HCl, pH 8.0, the protoxin activated with trypsin (1:50 w/w) for 2 h at 37 °C, and the reaction stopped by adding trypsin inhibitor (Sigma). The molecular mass and integrity of the recombinant protein were determined by SDS-PAGE.

2.3. Bioassays with the recombinant Cry1Ba6 protein

Five doses of recombinant Cry1Ba6 protoxin purified by centrifugation in sucrose gradients (500 μg/mL, 250 μg/mL, 100 μg/mL, 50 μg/mL and 25 μg/mL) were separately added to *A. grandis* artificial diet as described by Martins et al. (2007). Each protein dose was added to 5 mL of the artificial diet before it was poured out into six well plates (TPP). Five holes were punched in each well and each hole received a single neonate larva, in a total of five larvae per dose. The bioassay was kept in an incubator with photoperiod of 14 h/10 h (light/dark) at 27 °C. Mortality was recorded seven days later, and the LC_{50} obtained by Probit analysis (Finney, 1971). The bioassays were repeated three times and the LC_{50} were compared by ANOVA using the Sigma stat program (Kuo et al., 1992).

2.4. Preparation of BBMV of A. grandis

BBMV were prepared from dissected midguts of fourth ínstar larvae of *A. grandis* by differential precipitation using $MgCl_2$, as previously reported by Wolfersberger et al. (1987), and stored at −70 °C until use.

2.5. Binding assays with isolated BBMV

Recombinant Cry1Ba6 toxin was biotinylated using biotinyl-N-hydroxysuccinimide ester (GE Healthcare) following the manufacturer's instructions. Binding of 10 nM labeled toxin to 10 μg of

A. grandis BBMV total protein was carried out in 100 μl binding buffer [1× PBS, 0.1% BSA, 0.1% Tween 20, and pH 7.6]. After 1 h at 25 °C, unbound toxin was removed by centrifugation (10 min at 14,000 × *g*). The pellet containing the BBMV with bound toxin was washed twice with 100 μl of the same buffer and finally suspended in 1× PBS, pH 7.6. An equal volume of 2× sample loading buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue) was added, samples were boiled 3 min, the proteins separated in a SDS–PAGE gel (10%) and transferred to a nitrocellulose membrane using the Trans-Blot® SD – Semi Dry Transfer Cell (Bio Rad), according to the manufacturer's instructions. The biotinylated protein was visualized by incubation with streptavidin conjugated with peroxidase (ECL, GE Healthcare) (1:4000 dilution) for 1 h, followed by incubation with luminol (ECL, GE Healthcare). For competition assays, 10 nM labeled toxin plus 1000 nM unlabeled toxin were incubated with 10 μg of *A. grandis* BBMV total protein in 100 μl binding buffer.

2.6. Purification of GPI-anchored proteins and Cry1Ba6 binding

A. grandis BBMV (1.5 mg) were suspended in 500 μl of PBS2 [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 (pH 7.2)]. PIPLC (phospholipase-C) (3 units) from *B. cereus* (Boehringer Mannheim) was added to the BBMV suspension and incubated for 90 min at 30 °C, as previously described (Fernández et al., 2006). Membranes were recovered by centrifugation (11,000 × *g*, for 20 min), and the supernatant was analyzed for the presence of GPI-anchored proteins by SDS-PAGE, as control, the BBMV fraction (pellet) was incubated with Cry1Ba toxin. The Cry1Ba6 toxin was biotinylated using biotinyl-N-hydroxysuccinimide ester (Amersham Biosciences) as described above. A matrix of agarose–streptavidin (200 μl; Sigma–Aldrich) was incubated with 7 μg of biotinylated-Cry1Ba6 toxin overnight at 4 °C. After incubation, the matrix was washed ten times in 1× PBS2 to remove any unbound protein. The solution containing GPI-anchored proteins (42 μg) was incubated with the 200 μl of Cry1Ba6–agarose matrix. The matrix was washed four times with 1× PBS2. The binding proteins were then eluted using 200 μl of 1× PBS2, pH 9.5. The pH was adjusted to 7.2 and the samples were stored at 4 °C in the presence of protease inhibitor (PMSF 1 mM) (Fernández et al., 2006).

2.7. ALP (alkaline phosphatase) and APN (aminopeptidase-N) specific activities

Specific ALP and APN enzymatic activities were measured according to Fernández et al. (2006). Using 1 mg/ml of p-nitrophenyl phosphate and leucine-p-nitroanilide (Sigma–Aldrich) as substrates respectively, BBMV proteins (5 μg) were mixed with ALP buffer [0.5 mM $MgCl_2$, 100 mM Tris/HCl (pH 9.5)] containing 1.25 mM of p-nitrophenyl phosphate or APN buffer [0.2 M Tris/HCl (pH 8), 0.25 M NaCl] containing 1 mM of leucine-p-nitroanilide. Enzyme activities were monitored as the change in the absorbance at 450 nm for 3 min at 25 °C.

3. Ultrastructural analysis of *A. grandis* midgut incubated with Cry1Ba6 recombinant protein

Early fourth-instar larvae were fixed in a solution containing 2.5% glutaraldehyde, 5 mM $CaCl_2$ and 5% sucrose in 0.1% sodium cacodylate buffer pH 7.3 at 4 °C overnight, dehydrated in series of ascending acetone concentrations (30–100%) and embedded in LRGold resin (Thorpe, 1999, Fournier and Escaig-Haye, 1999). Ultra thin sections were obtained in an ultramicrotome (Leika ultracut UCT), blocked with 1%(w/v) BSA in PBS for 1 h and incubated with a solution containing 30 nM of Cry1Ba6 biotinylated toxin. As control ultrathin sections were incubated with BSA or PBS in substitution to Cry1Ba6 toxin. The ultrathin sections were washed in PBS three times for 30 s, to eliminate unbound toxin, and then incubated for 1 h at 25 °C with Nanogold® streptavidin (⌀ = 1.5 nm/Invitrogen) (1:100) in PBS. The ultra thin sections were washed in water three times for 30 s, stained with uranyl acetate, lead citrate and observed in a JEOL® 1011C Transmission Electron Microscope.

To verify that the binding was specific, a blocking assay was carried out using Cry1Ba6 unlabeled toxin. The ultrathin sections were blocked with BSA 1% (w/v) in PBS for 1 h at 25 °C and incubated for 1 h at 25 °C with a solution containing 100 nM or 1000 nM unlabeled Cry1Ba6 toxin. The ultrathin sections were washed in PBS three times for 30 s and incubated with 30 nM biotinylated and activated Cry1Ba6 toxin for 1 h at 25 °C. The ultrathin sections were washed in PBS three times for 30 s, to eliminate unbound toxin, and then incubated for 1 h at 25 °C with Nanogold® streptavidin (⌀ = 1.5 nm/Invitrogen) (1:100) in PBS. The ultra thin sections were washed in water three times for 30 s, stained with uranyl acetate, lead citrate and observed in a JEOL® 1011C Transmission Electron Microscope.

4. Results

4.1. Cloning, sequencing and expression of a cry1B gene from strain S601

A 3.6 kb fragment amplified by PCR from S601 *B. thuringiensis* strain plasmidial DNA was cloned into the pGEM-T easy vector

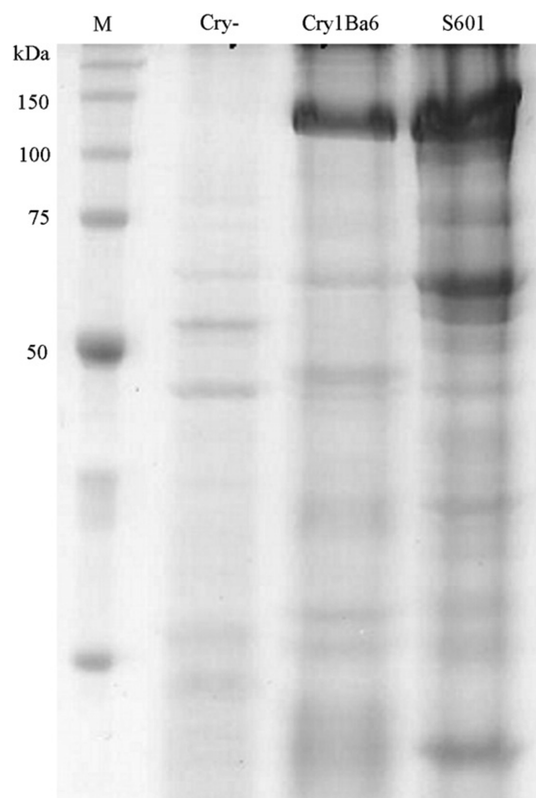

Fig. 1. Protein expression analysis in the recombinant *B. thuringiensis* strain. 12% SDS-PAGE showing in lane 1, the molecular mass marker, broad range ladder (Promega); lane 2, protein profile of the Bt Cry⁻ strain extract; lane 3, protein profile of the purified putative Cry1Ba6 crystals from the recombinant Bt strain containing the *cry1Ba6* gene; lane 4, protein profile of the purified crystals from the wild type strain S601 of *B. thuringiensis*.

Table 1
Bioassay against *A. grandis* using the Cry1Ba6 expressed by *B. thuringiensis* strain.

Strain	*N*	LC_{50} (μg/mL)[a]
S1122 (Bt)	25	380.8 (230.0–440.0)[b]
S601	25	140.2 (110.2–170.0)
Cry1Ba6	25	305.32 (288.2–320.4)

[a] The results are expressed as the means ± of three independent experiments.
[b] Number in parenthesis correspond to fiducial limits of 95%.

originating the pGEM1B601 (not shown) and completely sequenced (Genbank accession number EF102874). The analysis of the nucleotide composition of this gene revealed one ORF of 3687 bp, which potentially encodes a protein of 1228 amino acids. This sequence showed 98% amino acid identity to the *cry1Ba* gene described by Brizzard and Whiteley (1988) and was denominated *cry1Ba6*. The cry1B gene was removed from the pGEM1B601 plasmid and cloned into the *B. thuringiensis* expression vector pSVP27A (not shown). The pSVP1B601 recombinant plasmid was introduced into an acrystalliferous *B thuringiensis* subsp. *israelensis* IPS78/11 and the Cry1Ba6 recombinant protein expression analyzed by SDS-PAGE.The SDS-PAGE of purified recombinant Cry1Ba6 crystals (Fig. 1) showed a 140-kDa polypeptide band that was also present in the wild type S601 strain samples. This polypeptide was absent in the Bt cry^- strain extracts. All experiments were performed using purified recombinant Cry1Ba6 crystals.

4.2. Toxicity bioassay using the recombinant Cry1Ba6 protein against A. grandis

First instar larvae of *A. grandis* were incubated separately in artificial diet containing different doses of the Cry1Ba6 protein. After one week of exposure, the LC_{50} was determined. Cry1Ba6 showed LC_{50} values higher than the wild type S601 strain, but similar to the S1122 strain, which was used as a reference of a coleopteran-active isolate (Hofte et al., 1987) (Table 1).

4.3. Binding assay and homologous competition

Since we showed that the Cry1Ba6 recombinant protein was toxic to *A. grandis* we decided to analyze the binding of the toxin to the midgut of *A. grandis* larvae. We isolated BBMVs from the midgut of the *A. grandis* and incubated them with biotinylated-Cry1Ba6 recombinant protein. The binding assay showed that the toxin binds to different proteins of the BBMVs (Fig. 2A) and that the binding was specific since no binding was detected in the presence of a 100-fold excess of unlabeled Cry1Ba6 toxin (Fig. 2B).

4.4. Analysis of GPI-anchored proteins that interact with Cry1Ba6

In order to determine if GPI-anchored ALP or APN proteins are involved in the binding of the recombinant Cry1Ba6 toxin, biotinylated-Cry1Ba6 proteins were first used in a ligant blot assays with *A. grandis* BBMVs (10 μg), BBMVs treated wth PIPLC (10 μg)and affinity-purified proteins from BBMVs (Fig. 2C). The biotinylated-Cry1Ba6 protein was shown to bind to at least three proteins of 62, 65 (Fig. 2) and 100 kDa (Fig. 2B) but did not bind to the BBMV pellet remaining after PIPLC treatment.

Some Cry1 toxins have been shown to bind GPI-anchored ALP or APN receptors in the midgut of susceptible insects (Pigott and Ellar, 2007; Fernández et al., 2006). We determined ALP and APN activity profiles of affinity chromatography fractions containing the 62- and 65-kDa BBMV proteins that were treated with PIPLC and bound to immobilized Cry1Ba6 and compared activities with BBMVs and supernatants from BBMVs treated with PIPLC. ALP and APN activity were both detected in BBMVs and in the sample obtained after PIPLC treatment, which was expected to release GPI-anchored proteins such as ALP and APN. Proteins obtained by affinity chromatography only had ALP activity (Table 2), indicating that ALP could be one (or both) of the 62- or 65-kDa proteins from *A. grandis* BBMVs. However, there is a possibility of binding of biotin-like BBMV proteins to the resin used for purification of biotinylated-Cry1Ba6 protein since it that was not blocked.

4.5. Ultrastructural analysis of A. grandis midgut incubated with the Cry1Ba6 recombinant protein

To confirm the binding of the Cry1Ba6 toxin to the BBMVs of *A. grandis* we incubated ultrathin sections of midguts from *A. grandis* larvae with biotinylated-Cry1Ba6 protein followed by incubation with gold-streptavidin conjugate and observed in an electron microscope (Fig. 3I). The treatment of the gut tissue with

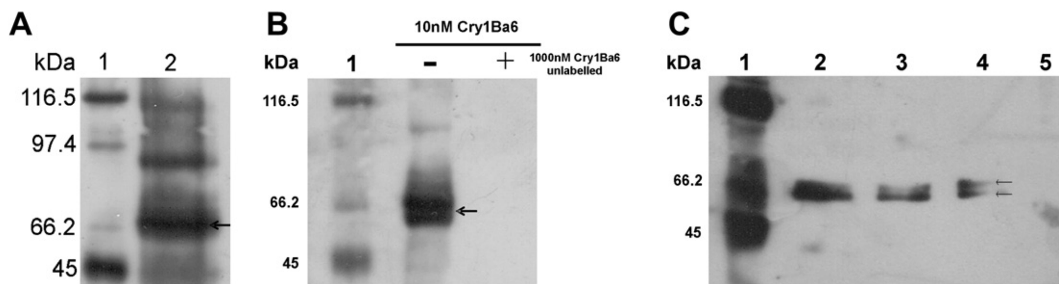

Fig. 2. Binding assays on BBMV isolated from *A. grandis*. A: One hundred μg of *A. grandis* BBMVs were separated by electrophoresis in an SDS-PAGE 9% gel and the proteins blotted onto a nitrocellulose membrane. This membrane was incubated with 10 nM biotinylated trypsin-activated Cry1Ba6 toxin and labeled protein was visualized by incubating the membrane with streptavidin–peroxidase conjugate and Luminol. Lane 1 biotinylated protein marker (Biorad), Lane 2 biotinylated trypsin-activated Cry1Ba6 bound to *A. grandis* BBMVs. Arrow indicate the binding of the biotinylated Cry1Ba6 to a protein of around 65 kDa protein (the ECL film was exposed for 15 min); B: Homologous competition, biotinylated trypsin-activated Cry toxins were incubated with the BBMV in the absence or in the presence of a 100-fold excess of unlabeled toxin. After 1 h post-incubation, unbound toxins were removed, and vesicles containing bound toxins were loaded onto an SDS-PAGE gel and blotted onto a nitrocellulose membrane. Labeled proteins were visualized by incubating the membrane with streptavidin–peroxidase conjugate and Luminol. Lane 1 biotinylated protein marker (Biorad), Line 2 biotinylated Cry1Ba6 (10 nM) without competitor, lane 3, biotinylated Cry1Ba6 (10 nM) + 1000 nM of Cry1Ba6 (the ECL film was exposed for 12 min). C: characterization of *A. grandis* GPI-anchored protein involved in binding Cry1Ba6 toxin. Ligand blot showing binding Cry1Ba6 (10 nM) to BBMV (10 mg), in solution. Lane 1: biotinylated protein marker (Biorad); lane 2: biotinylated Cry1Ba bound to BBMV proteins; lane 3: biotinylated Cry1Ba bound to BBMV proteins, previously treated with PIPLC and lane 4: biotinylated Cry1Ba bound to proteins obtained from Cry1Ba ligand chromatography and lane 5 BBMV proteins fraction remaining after PIPLC treatment (pellet) incubated with Cry1Ba toxin used as control. An arrow shows the position of the 65 and 62 kDa proteins (the ECL film was exposed for 7 min). The difference binding profile shown in Figs. 2A, 3B and 2C were due to the difference time of expose of each membrane.

Table 2
Specific ALP and APN activities of BBMV proteins.

Sample	ALP activity ($nM\,mL^{-1}\,min^{-1}/\mu g$)	APN activity ($\mu mL^{-1}\,min^{-1}/\mu g$)
BBMV	113.88 ± 15	51.38 ± 21
BBMV/PIPLC	130.83 ± 16	153.85 ± 38
Cry1Ba6 ligand chromatography	194.44 ± 32	0.01

The results are expressed as the means ± SD of three independent experiments.

biotinylated-Cry1Ba6 toxin and streptavidin-conjugated gold particles showed toxin binding to the apical microvilli of midgut cells (Fig. 3.IC). The ultrathin sections incubated with biotinylated BSA and incubated with only PBS, used as a control instead of biotinylated Cry1Ba6 showed no binding (Fig. 3.IA and IB).

A blocking assay by immunolabelling, confirmed the results obtained by homologous competition test, showing that the binding of Cry1Ba toxin to *A. grandis* microvilli is specific since, when the sections were incubated with unlabeled Cry1Ba toxin at 100 nM we also observed binding of labeled toxin (Fig. 3.IIA), but when incubated with 1000 nM of unlabeled Cry1Ba toxin, the binding was no longer detected (Fig. 3. IIB).

5. Discussion

The *cry1B* gene was cloned and sequenced for the first time in 1988 by Brizzard and Whiteley (Brizzard and Whiteley, 1988) but its insecticidal activity was not described. Hofte and Whiteley (1989) revealed that the *cry1B* gene encoded a protein of 140 kDa, which shows some identity with the C-terminus region of Cry1 toxins. The N-terminus region shows similarities with proteins of the Cry3 family (Bradley et al., 1995), suggesting that this Cry protein might have activity against insects of the orders Lepidoptera and Coleoptera. Bradley et al. (1995) using selective bioassays, demonstrated the Cry1B protein was toxic against *Phthorimaea operculella* (Lepidoptera: Gelechiidae), *Artogeia rapae* (Lepidoptera: Pieridae), *Plutella xylostella* (Lepidoptera: Plutellidae) *Trichoplusia ni* (Lepidoptera: Noctuidae), *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) and *Chrysomela scripta* (Coleoptera: Chrysomelidae). Zhong et al. (2000) demonstrated the activity of the Cry1Ba1 toxin against *C. scripta* and *Manduca sexta*. Arrieta et al. (2004) reported toxicity against *Hypothenemus hampei* (Coleoptera: Scolytidae). Nathan et al. (2006) against *S. frugiperda* (Lepidoptera: Noctuidae) larvae and finally Martins et al. (2007) demonstrated the *cry1B* gene was present in Bt strains active against *A. grandis* and proposed that this protein might be involved in the toxicity against this insect pest.

In this work, the *cry1B6* gene from the *B. thuringiensis* strain S601 was cloned and expressed in an acrystaliferous Bt strain. The sequence of this gene revealed 98% identity with the *cry1Ba* gene described by Brizzard and Whiteley (1988) (Genbank X06711). The Cry1Ba6 protein was tested against neonate *A. grandis* larvae and showed similar toxic activity when compared to the most known Bt strain (Btt) active against coleopteran insects. However, the Cry1Ba6 toxin showed a LC_{50} higher than the wild type S601 strain. This result indicates that there are other toxic proteins in S601 strain, which improve the toxic activity of this strain.

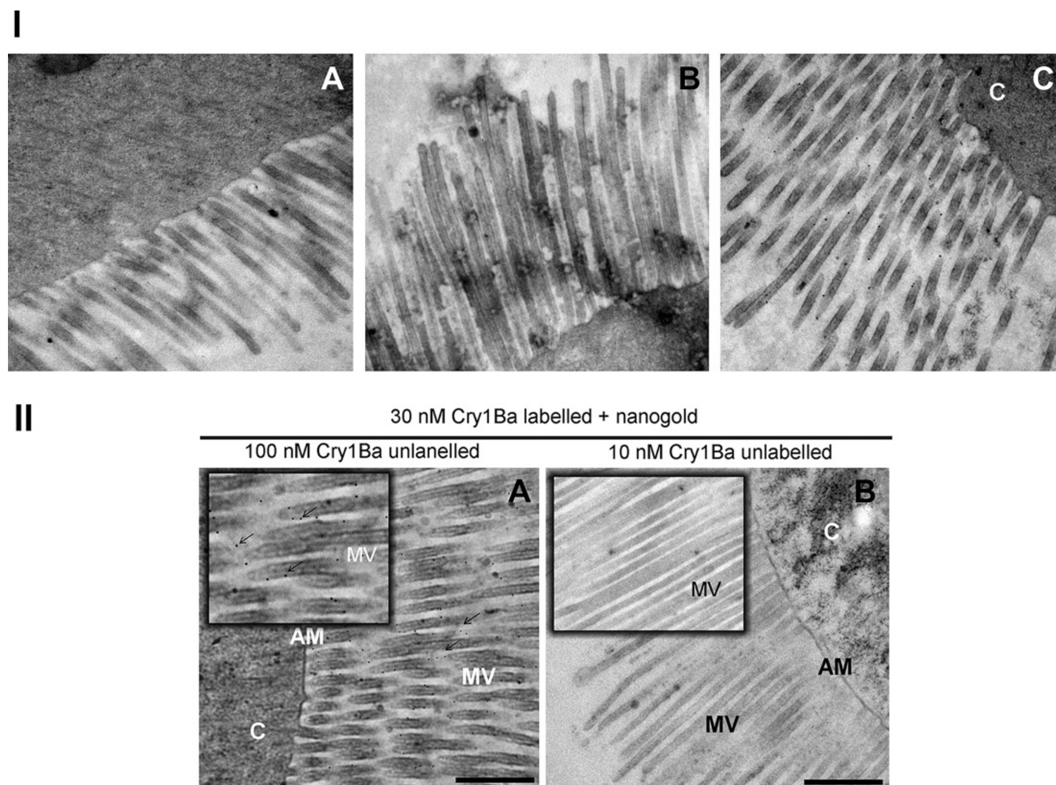

Fig. 3. Binding of Cry1Ba6 toxin to microvilli of *A. grandis*. I, Electron micrographs of ultrathin sections of *A. grandis* midguts treated with gold-streptavidin conjugate, which recognizes biotinylated proteins. A, midgut cells of *A. grandis* treated with 30 nM biotinylated BSA and Nanogold® streptavdin (∅ − 1.5 nm/Invitrogen); B, midgut cells of *A. grandis* treated with PBS 1X and Nanogold® streptavdin; C, midgut cells treated with 30 nM biotinylated Cry1Ba6 and Nanogold® streptavdin. MV, microvilli, C, midgut cell; MA, plasma membrane (bars = 500 nM). II, Blocking assay of Cry1Ba6 toxin to microvilli of *A. grandis*. Electron micrographs of ultrathin sections of *A. grandis* midguts treated with gold-streptavidin conjugate. A, midgut cells of *A. grandis* treated with 100-fold excess of unlabeled toxin and 30 nM biotinylated Cry1Ba6-Nanogold® streptavdin (∅ − 1.5 nm/Invitrogen); B, midgut cells of *A. grandis* treated 1000-fold excess of unlabeled toxin and 30 nM biotinylated Cry1Ba6-Nanogold® streptavdin. MV, microvilli, C, midgut cell; MA, plasma membrane (Bars = 500 nM).

SDS-PAGE of purified crystals produced by the BtCry1Ba6 recombinant strain showed a molecular mass of 140 kDa and transmission and scanning electron microscopy of the purified crystals revealed its rhomboidal shape (data not shown). This result was markedly different from the crystal morphology of the Cry toxin produced by Bt S601 strain, which has a bipyramidal shape (Martins et al., 2007). This morphological change probably could be associated with the presence and association of other proteins during the formation and assembly of the crystal in the Bt S601 strain.

On the other side the expression of the *cry1B* in different crops such as rice (*Oryza sativa*), maize (*Zea mays*) or potato (*Solanum tuberosum*) conferred resistance against different pest as *Chilo suppressalis* (Lepidoptera: Crambidae), *Diatrea grandiosella, D. saccharalis* (Lepidoptera: Pyralidae), *S. frugiperda* and *Phthorimaea opercuella* (Lepidoptera: Gelechiidae) (Breitler et al., 2000; Bohorova et al., 2001; Meiyalaghan et al., 2006). The fact that *cry1B* gene has been successfully expressed in different Bt-crops and that this protein conferred resistance to the insect attack may indicate that the expression of *cry1B* gene in transgenic cotton plants (*Gossypium* sp.) could be useful for the control of *A. grandis* and *S. frugiperda*, two important pests of cotton.

In our binding assay the recombinant Cry1Ba6 toxin bound specifically to BBMV isolated from *A. grandis*. Previous research have shown that Cry proteins, after activation by proteases localized in the midgut of the target insect, bind to specific receptors localized in the apical microvilli of the midgut cells of susceptible insects of the orders Lepidoptera (Hofmann et al., 1988a,b), Coleoptera (Bravo et al., 1992) and Diptera (Hofte and Whiteley, 1989; Ravoahangimalala et al., 1993). The recognition of the Cry toxin by specific receptors is a determinant factor for the specificity and activity of the Cry toxin. Even more, the main mechanism of resistance to Cry toxins are mutations that affect toxin–receptor interaction (Ferré and van Rie, 2002; Fernández et al., 2008). These data has motivated research towards understanding the mechanisms of toxin recognition and binding (Monnerat and Bravo, 2000). The comprehension of the molecular basis of toxin-receptor interaction will be useful to develop new Cry toxins with novel specificities and improved toxic activities, contributing to the management of insect resistance in the field. Most of the binding studies have been conducted in lepidopteran larvae and Cry1 toxins (Pietrantonio and Gill, 1996). Few studies were done in coleopteran or in dipteran larvae (Belfiore et al., 1994; Soberón et al., 2007a,b). This work is the first report about the localization of Cry toxin binding proteins in the midgut of *A. grandis*.

Some Cry toxin receptors have been previously characterized (Pigott and Ellar, 2007). The best characterized among them is the APN receptor (Pacheco et al., 2009, Rajagopal et al., 2003; Gill et al., 1995; Gill, 1995; Knight et al., 1994; Sangadala et al., 1994) and the cadherin-like receptor (Jurat-Fuentes and Adang, 2006; Flanagan et al., 2005; Zhang et al., 2005; Hua et al., 2004; Tsuda et al., 2003; Hara et al., 2003; Gahan et al., 2001; Valaitis et al., 2001; Nagamatsu et al., 1998a,b; Vadlamudi et al., 1995) identified in lepidopterans. In nematodes, glycolipids are believed to be involved in Cry toxin interaction (Griffitts et al., 2005). Other putative receptors include ALP (Fernández et al., 2006; Jurat-Fuentes and Adang, 2006; Jurat-Fuentes and Adang, 2004), a 270-kDa glycoconjugate (Valaitis et al., 2001), and a 252-kDa protein (Hossain et al., 2004).

We showed here that in *A. grandis* mainly two proteins of 65 and 62 kDa, bind to Cry1Ba6 toxin. These proteins were purified by affinity-chromatography and we demonstrated that they have alkaline phosphatase activity. In dipteran and lepidopteran larvae other ALPs have been identified as Cry toxin receptors. That is the case of Cry4B and Cry11 toxins that bind two proteins of 65 and 62 kDa in *A. aegypti* BBMV (Buzdin et al., 2002) that later was reported to correspond to ALP proteins (Fernández et al., 2006). In the case of Cry1Ac, a 68-kDa GPI-anchored ALP was identified as a toxin receptor in *H. virescens* (Jurat-Fuentes and Adang, 2004). Similarly a 65-kDa GPI-anchored ALP that binds Cry1Ac was identified in *M. sexta* (McNall and Adang, 2003). Interestingly, ALP expression levels were reduced in a resistant strain of *H. virescens*, supporting its functional role as a cry protein receptor (Jurat-Fuentes and Adang, 2004).

In this work, we have for the first time shown that the Cry1Ba6 protein from *B. thuringiensis* is toxic for *A. grandis* larvae and that the toxin binds to two GPI-anchored proteins with alkaline phosphatase activity in BBMV from midgut cells of this insect pest. We are now trying to determine the protein sequence of these putative Cry1B toxin receptors in the midgut of *A.grandis*, which could help improve our understanding of the structure of these receptors and the mode of action of Cry toxins.

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