Unshared binding sites for *Bacillus thuringiensis* Cry3Aa and Cry3Ca proteins in the weevil *Cylas puncticollis* (Brentidae)

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**A B S T R A C T**

*Bacillus thuringiensis* Cry3Aa and Cry3Ca proteins have been reported to be toxic against the African sweetpotato pest *Cylas puncticollis*. In the present work, the binding sites of these proteins in *C. puncticollis* brush border vesicles suggest the occurrence of different binding sites, but only one of them is shared. Our results suggest that pest resistance mediated by alteration of the shared Cry-receptor binding site might not render both Cry proteins ineffective.

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*Cylas puncticollis* (Bohemian) (Coleoptera: Brentidae) is one of the major pests of sweetpotato (*Ipomoea batatas* (L.) Lam.) in Eastern Africa (Smit, 1997). The sweetpotato weevil larvae make tunnels in roots and stems causing extensive damage (Stathers et al., 2005) which can cause yield losses of up to 60–100% depending on the severity of the infestation (Chalfant et al., 1990). Chemical control is not effective enough due to the cryptic habit of the larvae (Reddy et al., 2012). Hence, the use of transgenic plants which express *Bacillus thuringiensis* proteins (Bt crops) can be an useful alternative to control *C. puncticollis* insect pest as they have been shown to effectively control stem borers, ear feeders and rootworms (Loseva et al., 2002; Walters et al., 2008). Nowadays, genetically modified sweetpotato plants expressing Cry3Aa, Cry3Ca or Cry7Aa proteins, which have been reported to be active against *C. puncticollis* (Ekobu et al., 2010), have been developed in order to control different sweetpotato weevils of the genus *Cylus* (Morán et al., 1998; Rukarwa et al., 2013a, 2013b).

The mode of action of Cry proteins from *B. thuringiensis* has been extensively studied, especially for lepidopteran-active Cry proteins (Bravo et al., 2007; Vachon et al., 2012; Adang et al., 2014), whereas much less is known for coleopteran-active Cry proteins (Hernández-Martínez et al., 2014; Ochoa-Campuzano et al., 2012; Rausell et al., 2004, 2007; Slaney et al., 1992). The proposed model starts after the ingestion of the crystals by susceptible insect larvae, followed by crystal solubilization and protease activation in the midgut environment. Finally, the toxic fragment binds, as a key step, to specific receptors on the brush border membrane of the midgut epithelium columnar cells and that leads to toxin insertion into the membrane producing lytic pores which causes cell lysis and insect death (Federici et al., 2010).

The study of the Cry binding site model can help to maintain the long-term efficacy of Bt-crops, since binding site alteration has been described as the basis of cross-resistance when different Cry proteins share the same binding site (Ferré and Van Rie, 2002; Ferré et al., 2008). Recently, the existence of common binding sites for three *B. thuringiensis* proteins, Cry3Ca, Cry3Bb, and Cry7Aa proteins to *C. puncticollis* brush border membrane vesicles (BBMV) has been proposed (Hernández-Martínez et al., 2014). Thus, from a resistant management standpoint, combinations of these three proteins do not seem to be suitable for development of Bt sweetpotato plants. However, there is no information available about Cry3Aa protein binding sites in *C. puncticollis*. For this reason, the aim of the present study was to assess whether Cry3Aa and Cry3Ca proteins share binding sites in this pest to predict possible cross-resistance patterns for these Cry proteins which have been already introduced separately into sweetpotato plants.
Cry3Aa and Cry3Ca proteins were obtained from the
*B. thuringiensis* strains BGSC-4AA1 (provided by ARS Culture
Collection) and BTS02109P (provided by Bayer CropScience, Gent,
Belgium), respectively. Cry3 protein solubilization and activation,
either with bovine pancreas trypsin (type I) (Sigma-Aldrich) or
bovine pancreas x-chymotrypsin was performed as described by
Hernández-Martínez et al. (2014). Processing of Cry3Aa protoxin
with either trypsin or chymotrypsin renders a single main polypeptide with a mass of about 55 kDa (Fig. S1). Similar results were
described previously, though a second fragment of about 49 kDa
was also described to occur together with the 55 kDa fragment
when Cry3Aa protoxin was processed in *vitro* with chymotrypsin
(Carroll et al., 1997; Martínez-Ramírez and Real, 1996). These differences could be attributed to differences in the experimental
conditions used.

Processing of the Cry3Ca protein with either trypsin or
chymotrypsin renders a fragment with a mass of about 53 kDa
(Rausell et al., 2004) (Fig. S1). Additionally, processing of Cry3Ca
protein by either *C. puncticollis* gut fluid or BBMV also rendered a
fragment with a mass of about 53 kDa (Martínez-Solís et al.,
2011).

Cry3 proteins (73 kDa) are considered as truncated versions of
the lepidopteran-active proteins (130 kDa) (Park et al., 2009).
However, to be active the Cry3 protoxins must be processed at the
N-terminal part of the protein (Carroll et al., 1989; Rukmini et al.,
2000). In general, it has been proposed that serine proteases such
as trypsin-like or chymotrypsin-like proteases are involved in the
processing of *B. thuringiensis* Cry protoxins (Carroll et al., 1989,
1997; Mohan and Gujar, 2003; Oppert et al., 1996). In the present
study, the N-terminal sequence of either trypsin or chymotrypsin-activated Cry3Ca proteins was determined as described by
Hernández-Martínez et al. (2014). Briefly, protein bands were cut
out from the membrane and sent for N-terminal amino acid
sequencing by the Edman method at the Alphalyse A/S, Odense,
Denmark, using an ABI Procise 494 sequencer. The N-terminal
sequence of the trypsin-activated fragments was SQGRI, corre-
spanding to the position 159, whereas the N-terminal sequence of
the chymotrypsin-activated fragment was TLRDG at the position
153. The N-terminal sequences of the trypsin- and chymotrypsin-
activated Cry3Aa proteins was described by Carroll et al. (1997)
and correspond to the aminoacid positions 159 (sequence
NPHSQ) and 162 (sequence SQGRI), respectively.

Previous studies (Slaney et al., 1992; Rausell et al., 2004) have
shown that some Cry3 proteins are able to bind to BBMV from
some coleopteran insect pest including *C. puncticollis* (Hernández-
Martínez et al., 2014). Interestingly, some reports have shown
that only the chymotrypsin-activated Cry3Aa, and not the trypsin-
activated, was able to bind specifically to BBMV from *L. decemlineata* (Martínez-Ramírez and Real, 1996). In contrast,
Rausell et al. (2004) did not observe differences in the binding
ability of either trypsin- or chymotrypsin Cry3Aa protein to BBMV
from the same insect species. To clarify the active binding fragment
for Cry3Aa and Cry3Ca proteins in *C. puncticollis*, competition as-
says were carried out with either trypsin- or chymotrypsin-activated
proteins. BBMV were prepared from whole last-instar
*C. puncticollis* larvae based on the differential magnesium precipi-
tation method (Wolfersberger et al., 1987) as modified by Escriche
et al. (1995). Trypsin- and chymotrypsin-activated Cry3 proteins
were biotinylated with a protein biotinylation kit (GE HealthCare)
according to the manufacturer’s instructions. The working condi-
tions for the binding experiments were set up in preliminary experi-
ments. Competition experiments were performed incubating 5 µg of BBMV with 18 nm of biotinylated trypsin or chymotrypsin-
activated Cry3 proteins in binding buffer (phosphate-buffered sa-
line, pH 7.4, 0.1% BSA) in the absence or the presence of an excess of
unlabeled Cry proteins. Incubations were carried out for 1 h at 25 ºC
in a final volume of 100 µl. Moreover, control binding assays con-
ducted without BBMV showed practical absence of protein precipi-
tation (Fig. S2).

At least three replicates were performed to each competition
assay. Binding was detected as previously described by Hernández-
Martínez et al. (2014) using streptavidin-conjugated horseradish
peroxidase (1:2000).

Homologous competition assays showed that either trypsin or
chymotrypsin-activated Cry3Aa and Cry3Ca proteins bound speci-
fically to the *C. puncticollis* BBMV since they exhibited competi-
tion with an excess of their respective unlabeled Cry protein (Fig 1).
In order to test the role of proteolytic processing by commercial
enzymes on binding ability, labeled Cry3Aa and Cry3Ca trypsin
or chymotrypsin-activated proteins were competed with unlabeled
chymotrypsin-activated Cry3Aa or Cry3Ca proteins, respectively.
In all cases, the results showed a similar reduction on the binding of the biotinylated Cry3 proteins suggesting that independently of the protease treatment the Cry3 protein binds to the same receptor (Fig 1).

Thus, the differences in the N-terminal sequence described by
other authors to either trypsin- or chymotrypsin-activated Cry3Aa
(Carroll et al., 1997) or by ourselves to either trypsin- or

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![Fig. 1. Homologous competition binding assays on *C. puncticollis* BBMV. Biotinylated trypsin or chymotrypsin-activated Cry3 proteins were incubated in absence (−) or presence (250x) of unlabeled trypsin or chymotrypsin-activated proteins.](image-url)
proteins due to a single binding site modulation between Cry3Aa and Cry3Ca proteins. Demonstrated the occurrence of shared and unshared binding sites unlikely in BBMV (Rausell et al., 2004). Nevertheless, this is the three Cry3 proteins were also reported on Colorado potato beetle (Hernández-Martínez et al., 2011) and two Cry3 proteins (Cry3Bb and Cry3Ca) was previously described for mosquito control Open Toxinology J, 3, 83–85. http://www.ascrypt.com.

In summary, based on the results of binding site interactions, the development of cross-resistance between Cry3Aa and Cry3Ca proteins due to a single binding site modification appears to be unlikely in C. puncticollis, since both proteins have unshared binding sites. Thus, from a resistant management standpoint, combinations of Cry3Aa and Cry3Ca can be suitable for development of Bt sweetpotato plants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2016.09.014.

Transparency document

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