BINDING SITES FOR THE CRY1Ac INSECTICIDAL CRYSTAL PROTEIN OF *BACILLUS THURINGIENSIS* IN *HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE)

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Introduction

Concerns about the increase in resistance towards synthetic chemical insecticides in the cotton bollworm, *Helicoverpa armigera*, led the Australian cotton industry to adopt new pest management ideas. The introduction of INGARD® cotton containing a gene from the bacterium *Bacillus thuringiensis* (Bt) which expresses the Cry1Ac toxin is one such idea. This transgenic cotton will be followed up with another, BOLLGARD II®, which produces two Bt toxins, Cry1Ac and Cry2Ab. A major element of the introduction of transgenic plant technology will be to manage the risk of *H. armigera* becoming resistant to these insecticidal proteins.

Resistance to Bt has been recorded for at least 12 species of insect (Ferré and Van Rie, 2002). Although most of these species developed resistance under laboratory selection, resistance to Bt has been detected in many field populations of diamondback moth in various parts of the world. This resistance resulted from mismanagement of sprayable Bt formulations. Resistance to Bt toxins has most commonly been associated with alteration of a specific binding site that is required for the proteins to express their toxicity. The potential for *H. armigera* to develop resistance to Cry1Ac was demonstrated with the selection of an insect line with Cry1Ac (Akhurst *et al.*, 2000). The resistance in this *H. armigera* line was also linked to an alteration of the binding site, essentially the loss of the site.

A key element in the toxicity of the Cry insecticidal proteins is the binding of the toxin to a specific site on the midgut epithelium. Two families of proteins have repeatedly been identified as Cry toxin binding sites. These are aminopeptidase N (APN) and cadherin-like proteins (Francis *et al.*, 1997; Gill *et al.*, 1995; Lee *et al.*, 1996; Keeton *et al.*, 1998; Knight *et al.*, 1994; Simpson and Newcomb, 2000; Vadlamudi *et al.*, 1995; Valaitis *et al.*, 1995; Yaoi *et al.*, 1997). Both proteins are localised to the gut cell and are therefore plausible binding sites. Members of the APN family are also known to provide binding sites for viruses (Delmas *et al.*, 1992; Yeager *et al.*, 1992). The identity of the Cry1Ac binding site in *H. armigera* was investigated to assist in future resistance management initiatives.

Identification of Cry1Ac binding sites in H. armigera

Proteins that specifically bind Cry1Ac in *H. armigera* were isolated by affinity purification. Activated Cry1Ac was bound to a sepharose matrix and packed into a column. Membrane proteins from gut cells were passed through this column under conditions allowing proteins to bind Cry1Ac and be retained in the column. The proteins that bound to Cry1Ac were then recovered and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Six bands of binding proteins were detected (Figure 1) and analysed by mass spectrometer coupled to a nano flow HPLC to aid in identification of the component proteins. APNs were identified from five of the bands. A mixture of four different APNs were present in the group of five bands. The analysis of the sixth band showed some sequence similarity to a group of proteins not previously known to bind Cry1Ac.

Figure 1: Polyacrylamide gel of affinity isolated Bt target sites from *H. armigera*. Lane A- molecular weight markers in kilodaltons; Lane B- affinity purified proteins: bracket- five bands containing aminopeptidase N components; arrowhead- novel protein; # - Cry1Ac toxin (leaches off the column).

The genes corresponding to the identified *H. armigera* APNs were cloned by Polymerase Chain Reaction (PCR) and sequenced. They showed significant homology to other insect APNs (Table 1). The *H. armigera* genes HaAPN1, 3 and 4 have highest homology to APN genes from *Heliothis virescens*. These *H. virescens* genes are known to encode proteins shown to bind Bt. The *H. armigera* gene, HaAPN2, has low homology with a *Plutella xylostella* gene. The protein encoded by the PxAPN3 gene has not been tested as a Cry binding site.

Table 1. Gene sequence identity of *H. armigera* aminopeptidase Ns to other insect APN genes.

	Insects	Gene name	DNA Identity (%)	Reference:
HaAPN1	*H. virescens*	HvAPN170	85	Oltean *et al.*, 1999
	Manduca sexta	MSRNAAMIN	68	Knight *et al.*, 1995
	Bombyx mori	BmAPN	67	Yaoi *et al.*, 1999
HaAPN2	*P. xylostella*	PxAPN3	64	Nakanishi *et al.*, 1999
HaAPN3	*H. virescens*	HvAPN	84	Gill *et al.*, 1995
	Epiphyas postvittana	EpAPN	66	Simpson and Newcomb, 2000
HaAPN4	*H. virescens*	HvAPN110	87	Banks and Adang, 2001

Relevance of identified binding proteins to Cry1Ac toxicity

In *H. armigera* five potential targets for Cry1Ac were identified: the four members of the aminopeptidase N family and a novel binding protein. Research has previously confirmed that in *H. armigera* there is one important target site for Cry1Ac (Akhurst and Liao, 1996). Further investigation of *H. armigera* was required to determine which of the five targets is the functional receptor for Cry1Ac. The resistant line of *H. armigera,* which exhibited loss of the high affinity binding site (Akhurst *et al.*, 2000), was used in this investigation.

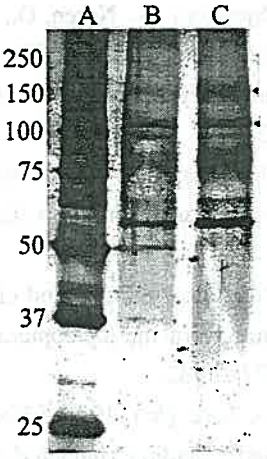

Figure 2: Polyacrylamide gel of affinity isolated Bt target sites from susceptible and resistant *H. armigera.* Lane A- molecular weight markers in kilodaltons; Lane B- affinity purified proteins from susceptible insects; Lane C- affinity purified proteins from resistant insects. Arrowheads- bands identified as APNs by mass spectrometry.

Cry1Ac binding proteins were purified from the resistant and susceptible strains of *H. armigera* on the affinity column and compared by SDS-PAGE (Figure 2). The protein profile from the resistant insects did not visibly differ from that of the susceptible insects. Mass spectrometry analysis provided good evidence that all four APN members were present in both the susceptible and resistant insects.

Conclusion

Five proteins were identified as possible Cry1Ac binding sites. These were four representatives of the aminopeptidase N family and a novel protein. However investigation of a resistant *H. armigera* strain, missing the Cry1Ac binding site, revealed no loss of any of the four APNs. Since the aminopeptidase Ns do not play a role in the resistance of *H. armigera* they can be eliminated as Cry1Ac binding sites. Determining relevance of the novel binding protein was hampered by ambiguous mass spectrometry data. Further investigation is required.

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