# Evidence for multiple mechanisms of resistance to Cry1Ac and Cry2A toxins from *Bacillus thuringiensis* in *Heliothis virescens*.

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*Bacillus thuringiensis* (Bt) is a common spore-forming bacterium that produces insecticidal proteins called Cry toxins (from Crystal). The commercialization of transgenic plants producing Cry toxins has greatly affected insect control methods due to their environmental safety and increased crop yield. In 1996, transgenic cotton plants producing Cry1Ac toxin were commercialized to control *Heliothis virescens* (tobacco budworm). This insect is one of the most important pests of cotton, among other crops. As with any insect control method, development of resistance to Bt toxins is one of the main concerns on the wide use of transgenic Bt plants. Although no *H. virescens* resistance episodes to Bt cotton have been reported in the field so far, laboratory

resistance selection of *H. virescens* has demonstrated that the genetic potential for resistance development exists (Gould *et al*., 1992, 1995). The study of resistance in these laboratory-selected insect strains helps to identify potential resistance mechanisms and develop strategies aimed to manage and delay the onset of resistance.

Disruption of any step in the mode of action of Bt toxins can result in resistance to these toxins. The general mode of action of Bt toxins includes ingestion by the susceptible insect, solubilization and activation to toxic forms by insect midgut enzymes, binding and insertion into the membrane of the midgut epithelium, and midgut cell lysis by osmotic shock (Knowles, 1994). Although several mechanisms of resistance to Bt toxins in laboratory-selected insects have been proposed, alteration of toxin binding to midgut receptors is the best studied (Ferré and Van Rie, 2002).

Since an insect is less likely to develop resistance to two toxins with distinct modes of action, one of the proposed methods to delay the onset of resistance to Bt plants in the field is the generation of transgenic lines expressing different Bt toxins in combination (Gould, 1998). To assure the efficacy of this approach the toxins selected for expression should not share common binding sites and must have distinct modes of action.

In brush border epithelium membrane vesicles (BBMV) from *H. virescens*, Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja toxins share a common binding site (receptor A), Cry1Ab and Cry1Ac have an additional binding site (receptor B) and Cry1Ac is the only toxin that can recognize a third binding site (receptor C) (Van Rie *et al*., 1989; Jurat-Fuentes and Adang, 2001). According to this model of binding sites, alteration of receptor A would potentially lead to reduced binding and possibly resistance

to all Cry1A, Cry1Fa and Cry1Ja toxins. This mechanism was proposed to occur in the Cry1Ac-selected YHD2 strain of *H. virescens* (Lee *et al*., 1995).

One of the most important toxin candidates to be used in combination with Cry1Ac in Bt cotton to control *H. virescens* is Cry2A. This toxin does not share binding sites with Cry1A toxins (Jurat-Fuentes and Adang, 2001) and has a distinct mode of action (English *et al*., 1994; Morse *et al*., 2001). Transgenic Bt cotton plants expressing both Cry1Ac and Cry2A have been shown to enhance control of *H. virescens* (Stewart *et al*., 2001).

Interestingly, the Cry1Ac laboratory selected CP73-3 and KCB *H. virescens* strains developed cross-resistance to Cry2A, among other toxins (Gould *et al*., 1992; Forcada *et al*., 1999). These strains were backcrossed to susceptible insects and the offspring were selected with Cry2A to increase resistance to this toxin. This selection regime led to the generation of the CXC (derived from CP73-3) and KCBhyb (derived from KCB) strains, which showed increased Cry2A and Cry1Ac resistance levels when compared to their parental strains (Kota *et al*., 1999). Both strains were also cross-resistant to Cry1Aa, Cry1Ab and Cry1Fa toxins (F. Gould, unpublished observation).

To study the mechanism of resistance in the CXC and KCBhyb strains, we performed toxin-binding assays with radiolabeled Cry1A toxins. BBMV from YDK (susceptible control strain), CXC and KCBHyb insects were isolated and incubated with increasing concentrations of labeled Cry1A toxins to generate a binding saturation curve for each Cry1A toxin. Saturation curves were analyzed and the binding affinities of each toxin for the CXC, KCBhyb and control susceptible BBMV were calculated. No changes in either toxin affinity or concentration of receptors were detected in BBMV from the

CXC strain when compared to susceptible vesicles. On the other hand, binding of Cry1Aa was greatly reduced in vesicles from KCBhyb, while Cry1Ab and Cry1Ac binding was as in BBMV from susceptible insects.

These results are evidence that resistance in the CXC strain is not due to changes in toxin binding to midgut receptors. Resistance in this strain should be the result of a change in a common step of the Cry1Ac and Cry2A toxin mode of action. Since these toxins seem to recognize different receptors in *H. virescens*, one possibility is alteration of steps prior to receptor binding in this strain. Such a change in the solubilization or processing of the Cry toxins in midguts of CXC insects would lead to resistance to both Cry1Ac and Cry2A. The existence of such a mechanism would be consistent with the decreased levels of susceptibility to other Bt toxins, as is the case for Cry1Aa, Cry1Ab and Cry1Fa.

Since Cry1Aa and Cry1Fa share a common binding site, we used biotinylated Cry1Fa (since iodination inactivates this toxin) to study binding of this toxin to BBMV from KCBhyb. No differences in Cry1Fa toxin binding were observed between YDK and KCBhyb, suggesting that binding of this toxin is not altered in KCBHyb larvae. Or at least, Cry1Fa binding is not altered to a degree detectable by the binding assay. Since Cry1Aa shares its only BBMV binding site with Cry1Ab, Cry1Ac and Cry1Fa, the change that is preventing Cry1Aa binding in KCBhyb BBMV is probably also responsible for resistance to all these toxins. This hypothesis was also proposed for the Cry1Ac resistant YHD2 strain of *H. virescens* (Lee *et al*., 1995) after obtaining the same qualitative toxin binding results we observed in KCBHyb BBMV. Additionally, since Cry1Aa and Cry2A do not share binding sites in *H. virescens* BBMV, cross-resistance to

Cry2A cannot be explained by alteration of Cry1Aa binding. In this case, a second mechanism of resistance that would affect both Cry1Ac and Cry2A mode of action needs to be present. As outlined for the CXC strain such a mechanism is may be related to alteration of toxin solubilization and/or processing conditions in the midguts of CXC and KCBhyb midguts.

In conclusion, our results indicate the presence of at least two resistance mechanisms in larvae from the KCBHyb strain. One of the mechanisms would be related to Cry1A receptor alteration, and possibly the second mechanism related to toxin solubilization and/or processing in the larval midgut. Similar conclusions have been presented for resistant *Plodia interpunctella* (Indianmeal moth) (Herrero *et al*., 2001). Alteration of toxin solubilization and/or processing seems to be the main mechanism of resistance in larvae from the CXC strain. Interestingly, high levels of Cry2A expression in chloroplasts of tobacco plants overcomes resistance in CXC larvae (Kota *et al*., 1999), indicating a possible solution to this resistance mechanism. Nevertheless, our conclusions raise questions as to how *H. virescens* in the field will respond to transgenic cotton producing Cry1Ac and Cry2A proteins. Our results are also evidence of the array of resistance mechanisms to Bt toxins that *H. virescens* can develop after selection with a single Cry toxin. This information is extremely important when designing and implementing strategies aimed at delaying resistance and cross-resistance to Bt transgenic crops

Experiments in our laboratory are presently aimed at elucidating the molecular mechanism by which decreased toxin binding is achieved in the KCBhyb resistant insects, as well as the molecular nature of the resistance mechanism in CXC larvae.

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