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Biochemistry

Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor

(insecticidal crystal protein/δ-endotoxin/diamondback moth)

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ABSTRACT **The biochemical mechanism for resistance to *Bacillus thuringiensis* crystal proteins was studied in a field population of diamondback moths (*Plutella xylostella*) with a reduced susceptibility to the bioinsecticidal spray. The toxicity and binding characteristics of three crystal proteins [CryIA(b), CryIB, and CryIC] were compared between the field population and a laboratory strain. The field population proved resistant (>200-fold compared with the laboratory strain) to CryIA(b), one of the crystal proteins in the insecticidal formulation. Binding studies showed that the two strains differ in a membrane receptor that recognizes CryIA(b). This crystal protein did not bind to the brush-border membrane of the midgut epithelial cells of the field population, either because of strongly reduced binding affinity or because of the complete absence of the receptor molecule. Both strains proved fully susceptible to the CryIB and CryIC crystal proteins, which were not present in the *B. thuringiensis* formulation used in the field. Characteristics of CryIB and CryIC binding to brush-border membranes of midgut epithelial cells were virtually identical in the laboratory and the field population.**

Insecticide resistance is a formidable practical problem in controlling agricultural insect pests and disease vectors. Insects have developed resistance to all major classes of chemical insecticides and it is likely that they will continue to do so, even with newly developed insecticides.

Bacillus thuringiensis is an environmentally sound alternative to the current chemical insecticides in the control of some phytophagous and vector insects. This Gram-positive bacterium produces crystalline parasporal inclusions containing insecticidal crystal proteins (ICPs), the so-called δ-endotoxins. Different strains produce crystal proteins with different insecticidal spectra (1). Due to their high specificity, ICPs are harmless to non-target insects, the environment, and end-users. *B. thuringiensis* insecticides have been used for >30 years but still represent <1% of all insecticides in use today. However, it is expected that during the coming years these bioinsecticidal sprays will be applied on a much larger scale. Crystal protein genes also have been transferred to plant genomes, resulting in transgenic plants protected from insect attack (2–5). The first transgenic insecticidal plants are expected to be marketed during this decade. To safeguard the future use of *B. thuringiensis* crystal proteins, it is of paramount importance to assess the extent to which insects are able to develop resistance to ICPs.

Laboratory selection experiments for resistance to *B. thuringiensis* have led to variable results (6–9). High levels of resistance to *B. thuringiensis* have been obtained by artificial selection only in the Indian meal moth, *Plodia interpunctella* (10). More recently, two cases of resistance development in the field have been reported. *B. thuringiensis* formulations have ceased to be effective in controlling the diamondback moth, *Plutella xylostella*, in an area of the Philippines exposed to this insecticide for many years (11). Also, some populations of this insect in Hawaii appear to have developed resistance (12).

Laboratory- and field-selected resistance may be due to different factors. Laboratory development of resistance is more likely to involve polygenes (multiple genes, each having a small impact on the selected trait), since they are prone to be selected under conditions where biological and environmental stress factors are minimized. In contrast, development of resistance in the field is more likely to involve single major genes, which can be selected from a much wider genetic pool under more stressful conditions (13).

It was recently demonstrated that the molecular basis of resistance in a laboratory-selected strain of *Plodia interpunctella* involved a change in a membrane receptor (14). However, it was unclear whether the mechanism of resistance identified in this strain was a consequence of the laboratory selection or whether it was exemplary for the natural mechanism of resistance to *B. thuringiensis* ICPs. For this reason we have investigated the mechanism of resistance in a field population of *Plutella xylostella* that had developed natural resistance to a *B. thuringiensis* insecticide.

MATERIALS AND METHODS

Insects and Toxicity Assays. The laboratory strain of *Plutella xylostella* was established several years ago from insects collected in the field in The Netherlands. The resistant population was established in the laboratory from pupae collected around Baguio, The Philippines, in an area that had been repeatedly exposed to Dipel (a commercial formulation of the *B. thuringiensis* var. *kurstaki* strain HD-1). Both insect populations were reared on fresh cabbage leaves, at 25°C, 60% relative humidity, and a 16 hr/8 hr photoperiod.

Toxicity assays were performed with third-instar larvae on a modified artificial diet (15). Freshly prepared diet was dispensed in wells of 2-cm^2 surface area (Costar, 24-well cluster plate). Five dilutions of the toxins were prepared in phosphate-buffered saline (PBS: 8 mM Na_2HPO_4/2 mM KH_2PO_4/150 mM NaCl, pH 7.4) containing 0.1% bovine serum albumin (BSA). The dilutions (50 μl) were applied uniformly over the food surface in each well and allowed to dry. Two larvae were placed into each well; 24 larvae were used per dilution. The multiwell plates were sealed with punctured plastic film. Mortality was scored after 5 days and

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Abbreviations: ICP, insecticidal crystal protein; BBMV, brush-border membrane vesicle; BSA, bovine serum albumin.

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the results were evaluated by probit analysis (16) and expressed as LC_{50} values (the concentration required to kill 50% of the insects).

***B. thuringiensis* ICPs.** The CryIA(b), CryIB, and CryIC *B. thuringiensis* crystal proteins were obtained as recombinant proteins expressed in *Escherichia coli*. Cloning of the gene for CryIA(b) from *B. thuringiensis* var. *berliner* 1715 has been described by Höfte *et al.* (17). The gene for CryIB was cloned from *B. thuringiensis* var. *entomocidus* HD-110. Its sequence is identical to that described by Brizzard and Whiteley (18). The gene for CryIC was cloned from *B. thuringiensis* var. *entomocidus* HD-110. This gene differs from the *cryIC* gene isolated by Honée *et al.* (19) only at the following positions: an Ala codon (GCA) instead of an Arg codon (CGA) is present at position 925 and a sequence of Thr-His (ACGCAT) instead of Thr-Asp (ACCGAT) is present at position 1400. Genes for CryID and CryIE were cloned from *B. thuringiensis* var. *aizawai* HD-68 (20) and *B. thuringiensis* var. *darmstadiensis* HD-146 (Plant Genetic Systems, unpublished), respectively. The toxic fragment of each ICP was obtained by trypsin digestion (17) and further purified by ammonium sulfate precipitation, streptomycin sulfate treatment, and anion-exchange chromatography (21).

Iodination Procedures. Trypsin-activated CryIA(b) was iodinated by the chloramine-T method as described by Van Rie *et al.* (22). Trypsin-digested CryIB and CryIC proteins were iodinated by the Iodo-Gen (Pierce) method as described by Hofmann *et al.* (23). Specific radioactivities were 1.7, 0.22, and 2.7 μCi/μg of protein (1 μCi = 37 kBq) for CryIA(b), CryIB, and CryIC, respectively.

Preparation of Brush-Border Membrane Vesicles (BBMVs). A transection was made behind the head and near the rear end of last-instar larvae submersed in ice-cold MET buffer (0.3 M mannitol/5 mM EGTA/17 mM Tris·HCl, pH 7.5). The midgut was pulled out of the dissected body and the malpighian tubules were removed using forceps. Midguts were rinsed in ice-cold MET buffer, frozen in liquid nitrogen, and stored at −80°C until required. The recovery was ≈1 g of midguts (wet weight) per 3300 larvae.

BBMVs were prepared according to the method of Biber *et al.* (24) as modified by Wolfersberger *et al.* (25). Midguts (0.4–0.8 g) were homogenized in 30–60 volumes of cold MET buffer with an electric blender. An equal volume of cold 24 mM $MgCl_2$ was added and the mixture was incubated on ice for 15 min. After centrifugation at 1600 × *g* for 15 min, the supernatant was transferred to another centrifuge tube and centrifuged again at 20,000 × *g* for 30 min. The pellet was suspended in MET buffer to a final volume of 2–4 ml. An equal volume of 24 mM $MgCl_2$ was added and the above protocol was repeated. The final pellet was suspended in 2 ml of 0.5 × MET buffer and distributed in 50- to 200-μl aliquots, which were immediately frozen and stored at −80°C until use. The yield was ≈5 mg of vesicle protein per gram of midgut.

Binding Assays. Immediately before use in the assays, BBMVs were thawed and spun in a microcentrifuge. The storage buffer was replaced with PBS/0.1% BSA. Duplicate samples of BBMVs and labeled crystal proteins were incubated in 100 μl of PBS/0.1% BSA at room temperature. After 30 min [for CryIA(b) and CryIB] or 90 min (for CryIC) the reaction was stopped by filtration through Whatman GF/F glass-fiber filters (previously soaked with PBS/0.5% BSA) in a Millipore sample collector (1225 unit). Filters were washed rapidly with 5 ml of ice-cold PBS/0.1% BSA and transferred to microtubes. The radioactivity retained in the filters was measured in a 1275 Minigamma counter (LKB). Reaction mixtures for experiments with various concentrations of BBMVs contained 0.4 nM CryIA(b), 12 nM CryIB, and 0.8 nM CryIC. For competition experiments the reaction mixture contained 25 μg of BBMV proteins and ≈0.4 nM labeled CryIA(b), or 12 μg of BBMV proteins and ≈0.8 nM labeled CryIC, plus various concentrations of unlabeled toxins.

Binding data were analyzed using the LIGAND computer program (26), which calculates the bound concentration of ligand as a function of the total concentration of ligand, given initial estimates of the affinity (K_d) and the binding-site concentration (R_t). Through an iterative process, the computer adjusts the values of K_d, R_t, and nonspecific binding until the curve generated by these parameters approximates the experimental curve as closely as possible. With this program it is possible to assess, on a statistical basis, which model gives the best representation of the experimental data (e.g., a one-site vs. a two-site model). A *t* test was used to determine whether the mean values of the binding characteristics measured were significantly different.

Determination of Protein Concentration. Protein concentrations of purified trypsin-activated crystal proteins were calculated from their absorbance readings at 280 nm (measured with a Uvikon 810P spectrophotometer), using an extinction coefficient (in $M^{-1}\cdot cm^{-1}$) of 76,050 for CryIA(b), 86,400 for CryIB, 75,650 for CryIC, 76,400 for CryID, and 69,900 for CryIE. The extinction coefficients were calculated based on the amino acid compositions of the different proteins. For labeled crystal proteins, protein concentration was determined by a "sandwich" ELISA technique (27). Primary antibodies were rabbit polyclonal antisera against the toxins, and second antibodies were mouse monoclonal antibodies. The conjugate used was alkaline phosphatase coupled to rabbit anti-mouse IgG (Sigma). *p*-Nitrophenyl phosphate (Sigma) was used as substrate, and the color intensity of the reaction was measured in a Titertek Multiskan MCC. Dilution series of unlabeled ICPs (in PBS/0.1% BSA) were used to estimate the protein content of the radioactive crystal protein samples.

Protein content of BBMVs was determined according to Bradford (28) with BSA as a reference.

RESULTS

Toxicity of ICPs to *Plutella xylostella*. Among the five Lepidoptera-specific activated *B. thuringiensis* ICPs tested, CryIA(b), CryIB, and CryIC were found to be toxic to the *Plutella xylostella* laboratory strain, although CryIC was less toxic than CryIA(b) and CryIB (Table 1). For the population reported to have developed resistance in the field to Dipel, high levels of resistance were found for CryIA(b) (>200 times higher compared with the susceptible strain), whereas the resistant insects proved as susceptible as the control insects to CryIB and CryIC (Table 1).

Binding of Iodinated ICPs to BBMVs. Iodinated CryIA(b), CryIB, and CryIC were incubated with various concentrations of vesicles from both strains (Fig. 1). The experiment with CryIA(b) showed a dramatic difference in binding to vesicles from the two strains. Maximum binding in the

Table 1. Toxicity of different trypsin-activated crystal proteins to the laboratory strain and the resistant strain of *Plutella xylostella*

	Laboratory			Resistant		
ICP	LC_{50}	FL_{95}	Slope	LC_{50}	FL_{95}	Slope
CryIA(b)	6.7	2.8–16.1	0.8	>1350	—	—
CryIB	1.2	0.8–1.7	3.1	2.3	0.7–5.3	2.6
CryIC	88.9	43.8–164.6	1.3	46.5	23.9–84.4	1.2
CryID	>1350	—	—	>1350	—	—
CryIE	>1350	—	—	>1350	—	—

Fifty percent lethal concentrations (LC_{50}), 95% fiducial limits (FL_{95}), and the slope were calculated by probit analysis (16). LC_{50} values of purified trypsin-activated crystal proteins are expressed as ng of protein per cm^2 of artificial diet.

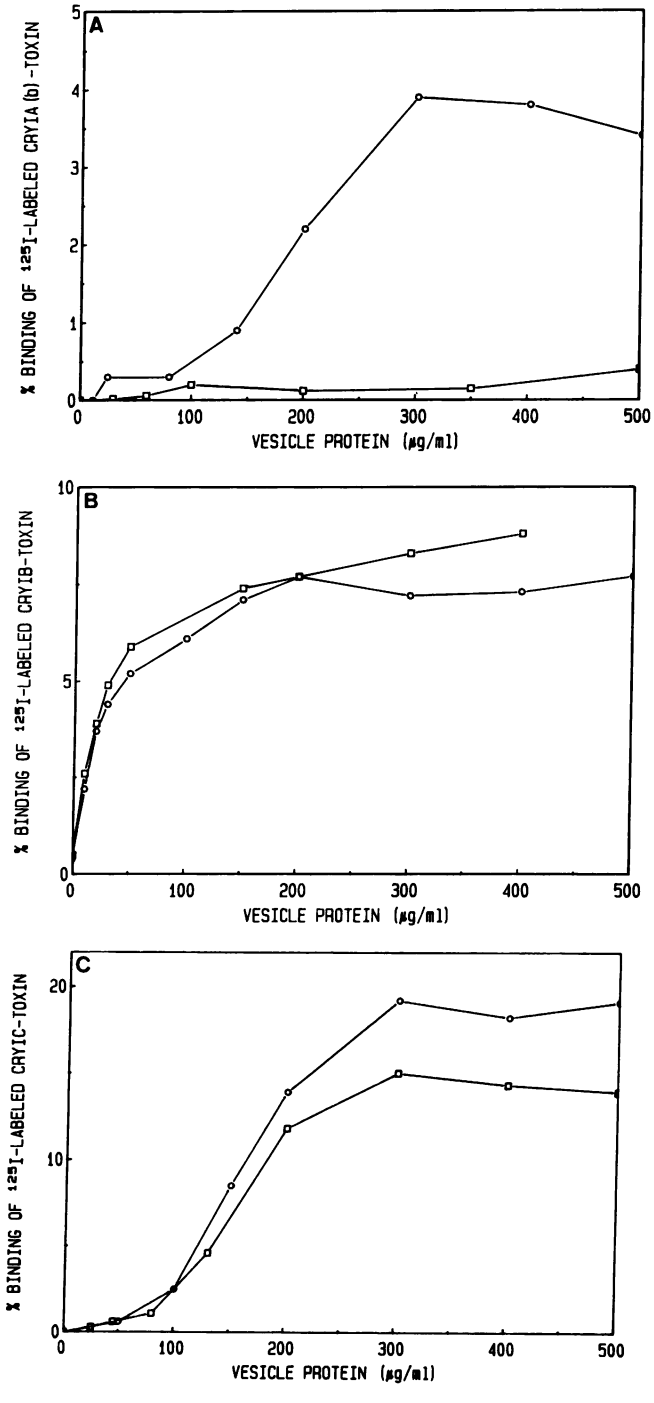

FIG. 1. Specific binding of ^{125}I-labeled CryIA(b) (*A*), CryIB (*B*), and CryIC (*C*) as a function of BBMV concentration. ○, Laboratory strain; □, resistant insects.

laboratory strain occurred at 300 µg/ml, whereas no binding could be observed in the resistant insects even at 500 µg/ml. On the other hand, no significant differences in binding were found for CryIB and CryIC. Maximum binding for these ICPs occurred at 200 µg/ml and 300 µg/ml, respectively. These results indicate that the *Plutella xylostella* laboratory strain contains specific receptors that bind CryIA(b), CryIB, and CryIC. In contrast, receptors for only CryIB and CryIC were detected in the resistant insects.

Competition Experiments. Homologous competition experiments (competition of a labeled ligand and its nonlabeled analogue for binding to the receptor) were performed with CryIA(b) and CryIC to obtain quantitative estimates of their binding characteristics for BBMV receptors (Figs. 2 and 3;

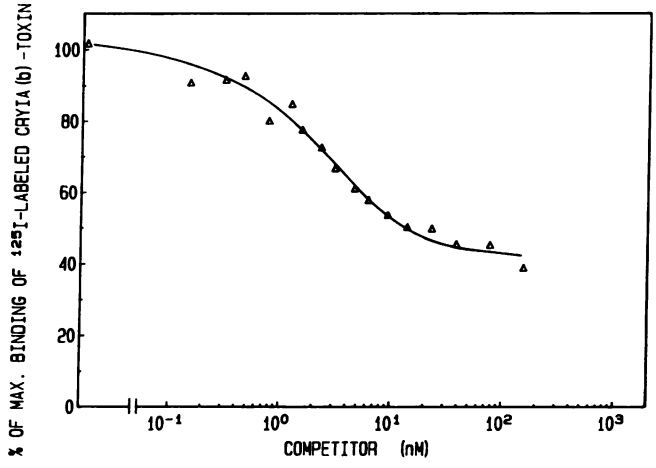

FIG. 2. Binding of ^{125}I-labeled CryIA(b) to BBMVs of the laboratory strain at increasing concentrations of nonlabeled CryIA(b) competitor.

Table 2). For binding of CryIA(b) to BBMVs of the laboratory strain, a dissociation constant (K_d) of 4.2 ± 2.5 nM and a receptor concentration (R_t) of 1.6 ± 1.2 pmol/mg of vesicle protein were calculated. Values for the characteristics of CryIA(b) binding to vesicles of the resistant insects could not be calculated because of the virtually complete lack of binding.

The two strains did not show significant differences in the binding parameters of CryIC (Table 2).

For CryIB we could not determine K_d and R_t values due to the low specific radioactivity of the labeled CryIB preparations.

An overall picture of the receptor system in *Plutella xylostella* was obtained by means of heterologous competition experiments. Fig. 3 shows that there is no competition of CryIA(b) and CryIB for the CryIC binding site. This indicates that CryIC recognizes a different receptor than CryIA(b) and CryIB. Furthermore, since the binding of CryIA(b) is dramatically different in the two strains whereas the binding of CryIB is similar in both, there must be different binding sites for both ICPs. In conclusion, for *Plutella xylostella* and these three ICPs, a model with three high-affinity binding sites can be proposed: one binding site specific for CryIC, another for CryIB, and a third one, which has been lost in the resistant insects, for CryIA(b).

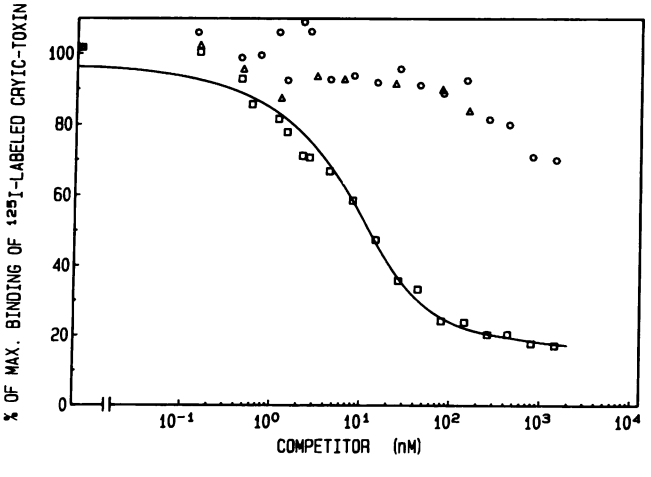

FIG. 3. Binding of ^{125}I-labeled CryIC to BBMVs of the resistant insects at increasing concentrations of nonlabeled competitor. △, CryIA(b); ○, CryIB; □, CryIC. (Essentially identical results were obtained when BBMVs of the laboratory strain were used.)

Table 2. Concentration of binding sites (R_t) and equilibrium dissociation constants (K_d) of *B. thuringiensis* crystal proteins on BBMVs of *Plutella xylostella*

ICP	Strain	K_d, nM	R_t, pmol/mg of BBMV protein
CryIA(b)	Laboratory	4.2 ± 2.5	1.6 ± 1.2
	Resistant	—	—
CryIC	Laboratory	6.5 ± 1.5	10.8 ± 7.3
	Resistant	7.6 ± 2.0	2.9 ± 0.3

Values are the mean (± SD) of three experiments for CryIA(b) and five for CryIC, performed on at least three independently prepared batches of vesicles.

DISCUSSION

Our aim was to investigate the biochemical mechanism of resistance to *B. thuringiensis* in a field population of *Plutella xylostella*. Therefore, we compared the toxicity and binding characteristics of different ICPs in two *Plutella xylostella* strains. The laboratory strain was established several years ago from a field population that to our knowledge had never been exposed to *B. thuringiensis*. The other population, obtained from a field in The Philippines, was reported to be resistant to Dipel, an insecticide based on a mixture of crystals and spores of *B. thuringiensis* HD-1. At present, it is unclear to what extent the *Plutella xylostella* population is resistant to Dipel in the field. We have tried to estimate the levels of resistance to Dipel, but in our hands, the field population proved equally susceptible to Dipel as the laboratory strain. However, we should stress that field conditions are very different from laboratory conditions and that different batches of Dipel may differ in crystal protein composition.

Five activated *B. thuringiensis* crystal proteins were tested for toxicity to *Plutella xylostella* larvae. Three crystal pro teins, CryIA(b), CryIB, and CryIC, showed significant levels of toxicity. Bioassays demonstrated that both field and laboratory insect populations were equally susceptible to CryIB and CryIC. In contrast, the field population was less susceptible to CryIA(b) by a factor of 200. It is important to note that Dipel contains CryIA and CryII crystal proteins, and no CryIB or CryIC ICPs. Therefore, it is reasonable to assume that the resistance to CryIA(b) has been induced by exposure to Dipel in the field. The insects are still susceptible to the CryIB and CryIC proteins, which demonstrates that in *Plutella xylostella*, resistance to CryIA(b) does not cause cross-resistance to CryIB and CryIC ICPs.

In most cases, a key determinant of the species specificity of the *B. thuringiensis* crystal proteins is the existence of specific receptors in the midgut of the insects (21, 22, 29). The resistance of a laboratory-selected *Plodia interpunctella* (Indian meal moth) strain is due to an alteration in a membrane receptor (14). Therefore, our study of the mechanism of resistance to crystal proteins has been focused on the receptors for these toxins in *Plutella xylostella*.

From our data we propose that *Plutella xylostella* possesses at least three different ICP receptors in the brush-border membrane of the midgut. No significant differences were found between the two strains with respect to CryIB and CryIC binding sites. However, the resistant strain has lost the capacity to bind CryIA(b). This might be due to either a decrease in receptor concentration, a decrease in affinity for the toxin, or both. We could not discriminate among these possibilities because there was no significant binding of CryIA(b) to BBMVs of the resistant strain.

This study has again shown that the toxicity of ICPs is dependent on binding to the midgut epithelial cells. Wolfersberger (30) reported an inverse correlation between ICP affinity and toxicity. In gypsy moth larvae, the CryIA(b) protein is some 400 times more toxic than CryIA(c) while its affinity is about 10 times lower. However, both proteins are bound with high affinity, with K_d values in the nanomolar range. In this study, we also showed that although CryIC is 10 times less toxic than CryIA(b) to the *Plutella xylostella* laboratory strain, both ICPs have comparable binding characteristics. It appears that the mechanism of action of crystal proteins is a two-step process: specific binding is followed by membrane disruption. This would mean that the toxicity is only in part determined by the binding characteristics. Specific binding seems essential for toxicity but is perhaps not sufficient. Possibly, some crystal proteins bind to insect cell membranes without being toxic. However, we postulate that an ICP cannot be toxic if it does not bind to the cell membrane.

In *Plutella xylostella*, as in *Plodia interpunctella*, resistance to *B. thuringiensis* is due to a change in one of the membrane ICP receptors. These insects have developed resistance in different environments: the *Plodia interpunctella* strain was selected for resistance in the laboratory, whereas resistance in *Plutella xylostella* had developed in the field. Nevertheless, the biochemical modifications responsible for resistance are the same in both species. This, along with the fact that membrane receptors play a key role in determining the specificity of *B. thuringiensis* ICPs, strongly suggests that this mechanism will probably apply to other instances of resistance to *B. thuringiensis* ICPs.

Knowledge of the physiology, biochemistry, and genetics of the development of resistance in insects to *B. thuringiensis* ICPs is essential to design effective strategies for management of pest resistance. This study has clearly demonstrated that insects can adapt to *B. thuringiensis* crystal proteins applied in the field, by changing their binding characteristics for the ICPs.

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