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CURRENT MICROBIOLOGY Vol. 49 (2004), pp. 22–27
DOI: 10.1007/s00284-003-4258-3

Current Microbiology
An International Journal
© Springer-Verlag New York, LLC 2004

Competition of *Bacillus thuringiensis* Cry1 Toxins for Midgut Binding Sites: A Basis for the Development and Management of Transgenic Tropical Maize Resistant to Several Stemborers

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Received: 13 October 2003 / Accepted: 24 November 2003

Abstract. Binding and competition of five *Bacillus thuringiensis* toxins—Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, and Cry1Ea—for midgut binding sites from three pests, *Spodoptera frugiperda, Diatraea saccharalis,* and *Diatraea grandiosella,* were investigated as part of a strategy to develop tropical transgenic maize resistant to several stemborers. On *S. frugiperda,* Cry1Ab and Cry1Ac compete for the same binding site; Cry1Ba and Cry1Ca compete for a second binding site. Cry1Ea recognizes a third specific binding site in *S. frugiperda* and does not compete with any of the other toxins. On *D. grandiosella* and *D. saccharalis,* Cry1Ac competes with Cry1Ab and not with Cry1Ba and Cry1Ca. Cry1Ba and Cry1Ca recognize each a specific binding site and do not compete with any of the other four toxins. Cry1Ea does not recognize any binding site on *Diatraea* species. Combinations of toxins are proposed to develop transgenic maize resistant to the three stemborers while allowing resistance management.

The bacterium *Bacillus thuringiensis* is characterized by the production of a crystalline inclusion body, also known as the "crystal," containing specific insecticidal crystal proteins or δ-endotoxins [8, 16]. The mode of action of the insecticidal crystal proteins involves a cascade of events including solubilization of the crystal, activation of the toxins by gut proteases, and recognition of a binding site on the midgut brush border membrane, followed by pore formation and cell lysis, leading ultimately to insect death [16]. These insecticidal crystal proteins, also known as Cry toxins, are the active compounds of all the commercially grown, insect-resistant transgenic plants.

Although not recorded yet on transgenic plants, insect resistance to Cry proteins has been documented in open fields on the diamondback moth after exposure to sprayable formulations [18] but also under laboratory conditions on a large range of insect species [5, 7, 17]. Several mechanisms of resistance have been described [5, 7], but the most frequently encountered mechanism is the modification of the binding sites [5, 19]. Numerous works have, therefore, been focused on the interaction between toxin and binding sites, which is responsible for specificity, and on the role of the modification of the binding sites on insect resistance [5]. The resistance management strategy recommended and currently implemented in the USA for insect-resistant transgenic plants is the expression of a high dose associated with the use of refuges [7]. Another strategy to delay evolution of resistance is the use of combinations of toxins recognizing different binding sites in the same insect species. This strategy, especially considered in association with the high-dose refuge strategy, is expected to prove very efficient.

Tropical and sub-tropical varieties of maize have been engineered with *B. thuringiensis* toxin genes for resistance to *Spodoptera frugiperda, Diatraea grandiosella,* and *Diatraea saccharalis* under a UNDP-funded

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project for release in Mesoamerican developing countries where yield losses to insects exceed 30% [1–3, 9]. Following the identification of toxins active against the selected insect species [1], the strategy for the deployment of transgenic varieties was to target several pests at once while allowing the implementation of resistance management through the stacking of toxins recognizing different binding sites in the three species of insect pest. We report here the analysis of the competition of five toxins for binding sites on *S. frugiperda, D. grandiosella,* and *D. saccharalis,* from in vitro binding assays with brush border membrane fractions (BBMF) and iodinylated toxins. Combinations of toxins highly active against the selected pests which recognize different binding sites are identified in order to help in implementing insect resistance management.

Materials and Methods

Insect pests and rearing conditions. *S. frugiperda, D. saccharalis,* and *D. grandiosella* were obtained from permanent colonies established at CIMMYT (El Batan, Mexico). Insects were reared on Mihm medium [13] as previously described [1] at 25°C, 65% relative humidity, and a photoperiod of 16:8 h (L:D).

Toxin production and purification. Cry1Ab was purified from a recombinant *B. thuringiensis* strain expressing the *cry1Ab* gene from *B. thuringiensis* HD-1 strain (these genes were kindly provided by L. Masson, Biotechnology Research Institute, Montreal, Canada). Cry1Ba, Cry1Ca, and Cry1Ea were purified from recombinant *B. thuringiensis* strains expressing the *cry1Ba* gene from *B. thuringiensis* strain HD-110, the *cry1Ca* gene from *B. thuringiensis entomocidus* strain 60.5 [20], and the *cry1Ea* gene from *B. thuringiensis kenyae* strain 4F1 gene [21], respectively. Cry1Ac was prepared from the native *B. thuringiensis* HD-73 strain. Bacterial strains were grown until complete lysis as previously described [15]. Cultures were harvested by centrifugation, and parasporal inclusion bodies were purified as described previously [11]. Protein concentration was determined by the method of Bradford [4]. Inclusion bodies were solubilized, and protoxins were activated as described previously [6]. Activated toxins were purified by low-pressure liquid chromatography through a Q-Sepharose anion exchange column (Pharmacia), as described previously [15]. Fractions containing the eluted protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and dialyzed for 48 h at 4°C against sterile, double-distilled water until protein precipitation and then lyophilized.

Protein labeling. Purity was checked by SDS-PAGE analysis, and protein concentration was determined by the Bradford dye-binding procedure [4] prior to iodinylation by the chloramin T method [12] as described elsewhere [6, 22]. Labeling and quality of the toxins was checked by SDS-PAGE followed by an autoradiography. Average of specific activity of all the labeled toxins was 100,000 cpm/pmol.

BBMF preparation. *S. frugiperda, D. saccharalis,* and *D. grandiosella* fertilized eggs were reared on artificial diet [13] until larvae reached the fifth instar. Midguts were then isolated from early fifth instar larvae. Dissected midguts were stored frozen without buffer at −80°C prior to BBMF preparation. Midgut brush-border membrane fractions (BBMF) were prepared from approximately 5000 isolated midguts as previously described [6, 22] by using selective precipitation by divalent cations and additional differential centrifugation steps. BBMF protein contents was determined by the method of Bradford [4] with the Bio-Rad protein assay dye reagent, and bovine serum albumin (BSA) as a standard. BBMF quality was assessed by testing for the apical membrane marker enzyme leucine aminopeptidase [10].

In vitro binding experiments. Binding experiments were performed in 1.5 mL poly(ethylene) microcentrifuge tubes, in 20 mM phosphate buffer pH 7.4 containing 0.15 M NaCl, 0.1% bovine serum albumin, and 0.02% NaN_3 (PBS/BSA/Az). Binding assays were performed at least twice in duplicate, with overnight incubation at room temperature, in a total volume of 100 μL, with 20 μg of BBMF protein. BBMF-bound toxin was separated from free toxin by centrifugation and retained radioactivity measured in a liquid scintillation counter as previously described [6, 22]. Saturation experiments (direct binding) were conducted by mixing various concentrations of labeled toxin (2, 8, 24, 50, 100, and 150 nM) with 20 μg of BBMF proteins. Non-specific binding was determined in parallel assays with labeled activated toxin and an excess (1 μM) of unlabeled activated toxin. Homologous and heterologous competition was assessed by incubating a 10-nM solution of labeled Cry toxins and 20 μg of BBMF protein with each of a series of concentrations (0, 1, 3, 10, 30, 100, 300, and 1000 nM) of unlabeled homologous or heterologous toxins. Binding data were analyzed with the LIGAND program [14] (Biosoft/Elsevier).

Table 1. Binding parameters of Cry1 toxins on BBMV from *S. frugiperda, D. grandiosella,* and *D. saccharalis*

Insect	Toxin	kDa (nM)	B_{max} (pmol/mg)
Spodoptera frugiperda	Cry1Ab	2.4	5.4
	Cry1Ac	1.8	6.3
	Cry1Ba	6.2	2.1
	Cry1Ca	2.1	10.1
	Cry1Ea	0.8	2.8
Diatraea grandiosella	Cry1Ab	3.4	8.4
	Cry1Ac	1.2	3.7
	Cry1Ba	0.6	4.0
	Cry1Ca	4.9	9.6
	Cry1EA	NB[a]	NB
Diatraea saccharalis	Cry1Ab	4.3	3.2
	Cry1Ac	1.6	5.1
	Cry1Ba	0.8	3.4
	Cry1Ca	5.6	8.9
	Cry1Ea	NB	NB

[a] NB, no binding.

Results

Direct binding experiments. Saturation binding assays showed that all five toxins, i.e., Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, and Cry1Ea, bound specifically to BBMFs from *Spodoptera frugiperda* (Table 1), whereas only four, i.e., Cry1Ab, Cry1Ac, Cry1Ba, and Cry1Ca, bound specifically to *D. grandiosella* and *D. saccharalis* (Table 1). Cry1Ea did not recognize any binding site on BBMFs from both *Diatraea* species (Table 1). The constants of affinity for the binding site, calculated from homologous competition, are presented in Table 1. The

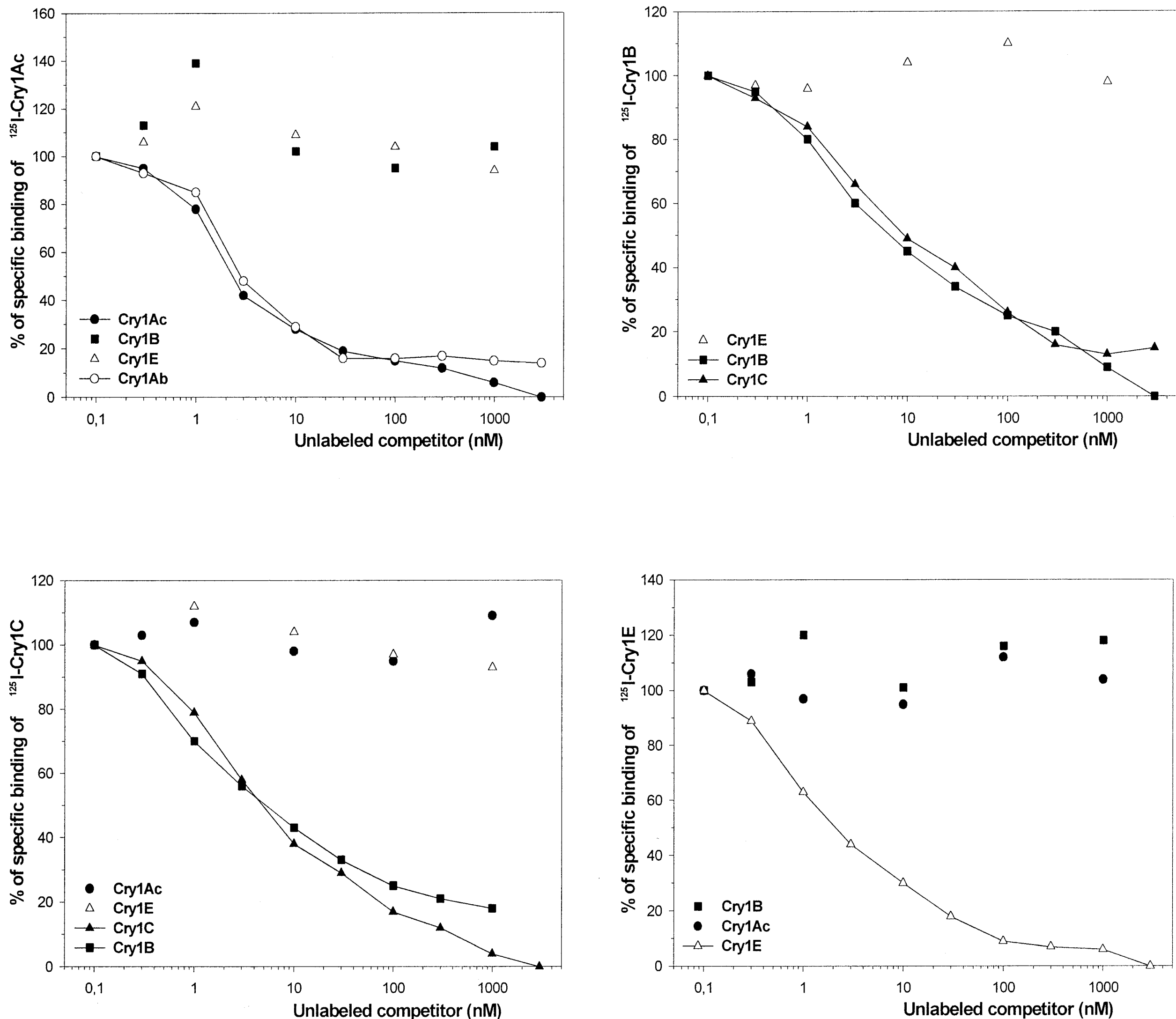


Fig. 1. Homologous and heterologous competition of ^{125}I-labeled CryI toxins to BBMV of *Spodoptera frugiperda.*

Scatchard plots (data not shown) were linear, showing that the toxins bound to a single class of receptors. Non-specific binding was assessed in all assays by adding excess of unlabeled toxin. Non-specific binding increased linearly with concentration of radiolabeled toxin and was about 50% at that of specific binding in these experiments.

Competition experiments. Binding analyses of Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, and Cry1Ea to BBMVs from *Spodoptera frugiperda* were conducted through a set of homologous and heterologous competitions. Such competitions, conducted with labeled and unlabeled Cry1Ac, showed that this toxin binds specifically to a binding site in *S. frugiperda* and that it is competing for this binding site with Cry1Ab (Fig. 1a). The binding site recognized by both Cry1Ac and Cry1Ab is not recognized by either Cry1Ba, Cry1Ca, or Cry1Ea. Similar analyses conducted with a labeled Cry1Ba toxin showed that the specific binding site recognized by Cry1Ba is also recognized by Cry1Ca. Cry1Ea does not compete for this binding site (Fig. 1b). This competition for binding site in *S. frugiperda* between Cry1Ba and Cry1Ca is confirmed by homologous and heterologous competitions conducted with a labeled Cry1Ca toxin (Fig. 1c). This also shows that the binding site shared by Cry1Ca and Cry1Ba is not recognized by Cry1Ac and Cry1Ea. Homologous and heterologous competitions performed with labeled Cry1Ea indicate that this toxin

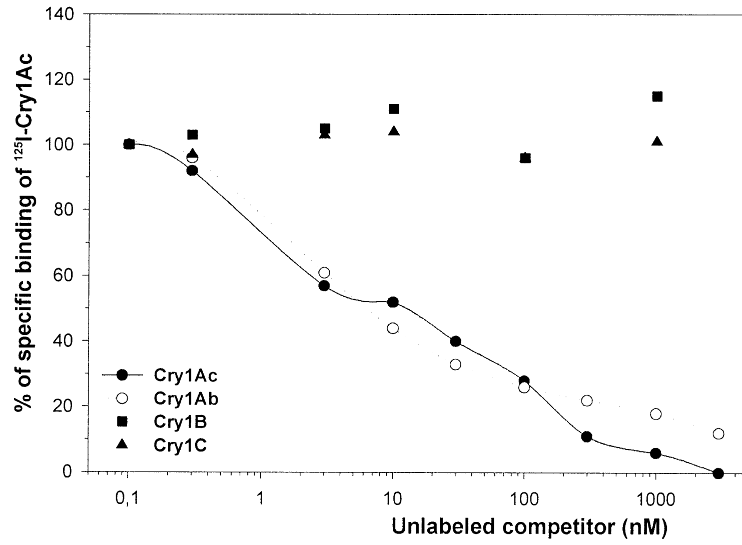
recognizes a specific binding site that is not recognized by Cry1Ac, Cry1Ba, and Cry1Ca (Fig. 1d). These data show that, in *S. frugiperda,* three different binding sites are recognized by the toxins tested. Cry1Ab and Cry1Ac are competing for the same binding site and do not compete with Cry1Ba, Cry1Ca, and Cry1Ea. Cry1Ca and Cry1Ba are competing for the same binding site and do not compete with Cry1Ab, Cry1Ac, and Cry1Ea. Cry1Ea recognizes a different binding site and does not compete with Cry1Ab, Cry1Ac, Cry1Ba, and Cry1Ca.

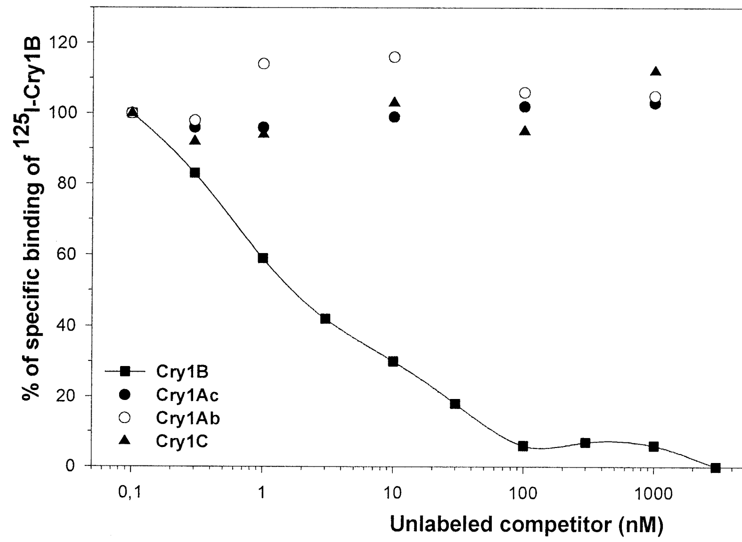
Similar analyses were conducted with the same toxins on BBMVs from *D. grandiosella* and *D. saccharalis.* The results obtained with the two *Diatraea* species were very similar and, therefore, with respect to susceptibility to *B. thuringiensis* toxins and to specificity of binding sites, these two distinct species can be considered as a single pest. As a consequence, only the results from homologous and heterologous competition experiments with BBMVs from *D. grandiosella* are illustrated in Fig. 2. Homologous competitions conducted with labeled and unlabeled Cry1Ea showed that, in both species, binding of labeled Cry1Ea in the presence of an excess of unlabeled toxin, i.e., nonspecific binding, is very high (data not shown). This result indicates that Cry1Ea does not bind to a specific receptor on BBMVs from *D. grandiosella* and *D. saccharalis.* Homologous and heterologous competition performed with labeled Cry1Ac (Fig. 2a) showed that Cry1Ac binds to BBMVs specifically. Competition was observed between Cry1Ab and Cry1Ac, whereas Cry1B and Cry1C toxins did not compete with labeled Cry1Ac. This indicates that Cry1Ab and Cry1Ac recognize and compete for the same binding site. Homologous and heterologous competitions (Fig. 2b) show that Cry1Ba binds specifically to BBMVs and that there is no competition with unlabeled Cry1Ca. A reverse experiment of homologous competition with labeled and unlabeled Cry1Ca (Fig. 2c) confirmed that Cry1Ca recognizes a specific binding site different from those recognized by Cry1Ba on the one hand, and by both Cry1Ab and Cry1Ac on the other hand. Binding data indicate that three binding sites are recognized by the various toxins tested on both species of *Diatraea.* As a summary, Cry1Ea does not recognize any specific binding site. Cry1Ab and Cry1Ac are competing for the same binding site and do not compete with Cry1Ba and Cry1Ca. Cry1Ba recognizes a specific binding site and does not compete with Cry1Ab, Cry1Ac, and Cry1Ca. Cry1Ca recognizes a specific binding site and does not compete with Cry1Ab, Cry1Ac, and Cry1Ba.

Discussion

The data reported in this article represent the basic information for the deployment of transgenic maize varieties to control tropical and subtropical maize stemborers in Mesoamerica. The results of competitive binding experiments described here indicate that the implementation of resistance management associating two highly active toxins recognizing different binding sites is clearly feasible for each of the three species of maize stemborers considered. The toxins active against *S. frugiperda,* i.e., Cry1Ab, Cry1Ca, and Cry1Ea [1], do not compete for binding sites; the following combinations could be used to help manage the evolution of resistance to *B. thuringiensis* toxins: Cry1Ca + Cry1Ab, Cry1Ca + Cry1Ea, Cry1Ab + Cry1Ea, and Cry1Ab + Cry1Ca + Cry1Ea. With respect to the control of *Diatraea,* Cry1Ba and Cry1Ab were the only toxins found to be active against both species of *Diatraea* [1–3]. Since these toxins do not compete for receptor binding, associating Cry1Ab and Cry1Ba is the obvious suitable combination for controlling these two pests with some level of resistance management. Transgenic tropical maize varieties expressing the *cry1Ab* and *cry1Ba* genes individually or as a translational fusion gene have been developed in order to assess the possibility of producing two different toxins in the same plant [3]. The insecticidal activity of the plants producing both toxins was shown to be high and to confer resistance to all three species of stemborers [3]. From the data reported here, this same transgenic tropical maize variety will also be suitable for implementation of a resistance management strategy for the control of *Diatraea.* The implementation of a resistance management strategy for the control of *S. frugiperda* would require the use of combinations associating Cry1Ab, Cry1Ca, and Cry1Ea. However, transgenic maize varieties expressing the *cry1Ea* gene displayed a limited insecticidal activity against *S. frugiperda* (CIMMYT, unpublished data) and, therefore, the most suitable combination should be that associating Cry1Ab with Cry1Ca.

As shown in Table 2, no combination of two toxins recognizes different binding sites and allows addressing the complex comprising the three species *S. frugiperda, D. saccharalis* and *D. grandiosella.* In this case, the solution will be to develop combinations of three toxins. Indeed, a combination associating in the same plant Cry1Ab, Cry1Ba, and Cry1Ca will provide effective control of the three pests and will deliver at once and for each pest two active toxins recognizing two different binding sites. *S. frugiperda* will be controlled by the non-competing toxins Cry1Ca and Cry1Ab, and both *Diatraea* species will be controlled by the non-competing toxins Cry1Ab and Cry1Ba.





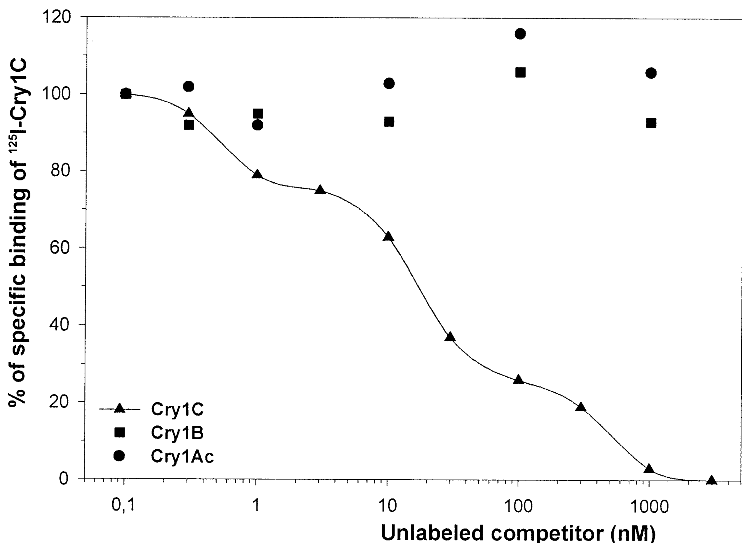


Fig 2. Homologous and heterologous competition of ^{125}I-labeled CryI toxins to BBMV of *Diatraea grandiosella*.

Table 2. Combinations of toxins suitable for resistance management on *S. frugiperda, D. grandiosella,* and *D. saccharalis*

Insect pests	Combinations of toxins
Spodoptera frugiperda	CrylCa + CrylAb
	CrylCa + CrylEa
	CrylAb + CrylEa
	CrylAb + CrylCa + CrylEa
Diatraea grandiosella	CrylAb + CrylBa
Diatraea saccharalis	CrylAb + CrylBa
Spodoptera frugiperda	CrylCa + CrylBa + CrylAb
Diatraea grandiosella	
Diatraea saccharalis	

An efficient release of insect-resistant transgenic maize plants for developing countries will require selection of suitable open-pollinated tropical varieties of maize, the selection of toxins highly active against local pests, the availability of a strategy for the simultaneous deployment of several genes in order to control a complex of pests, and the possibility to provide a minimal level of sustainability by allowing the use of resistant management. By addressing this last point, the work reported in this article will allow the sound deployment of optimized insect-resistant maize capable of meeting the local needs and demand and provide benefits to local farmers.

ACKNOWLEDGMENTS

This work was supported by UNDP Global Research and Development Project GLO/91/014 under management by CIMMYT (International Maize and Wheat Improvement Center).

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